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THE EFFECTS OF STORAGE ON IRRADIATED RED BLOOD CELLS: AN IN VITRO AND IN VIVO STUDY

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Capt, USAF, BSC

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THE EFFECTS OF STORAGE ON IRRADIATED RED BLOOD CELLS: AN IN VITRO AND IN VIVO STUDY

Susan E. Knoll

Submitted to the Graduate College of Bowling Green State University in partial fulfillment of the requirements for the degree of

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ABSTRACT

Irradiation of red blood cell units has recently become a topic of special concern as the result of increasing reports of graft versus host disease in immunocompetent blood transfusion recipients. This study was designed to evaluate the potassium elevations observed in stored irradiated red blood cells and to evaluate the *in vivo* survival of stored irradiated red blood cells using a dog model. In the *in vitro* study ten units of human CPDA-1 packed red blood cells were made into paired aliquots; one aliquot of each pair was irradiated with 3000 rads of gamma radiation and the potassium content measured at points throughout 35 days of storage. A significant increase in potassium levels in the irradiated aliquots was observed from the first day after irradiation and continued through the entire storage period. In the *in vivo* study red blood cells survival studies were performed on five dogs by transfusing irradiated stored autologous packed red blood cells labeled with a fluorescent dye. Red cell survival was not clearly affected by irradiation. All irradiated units had poorer 24 hour red cell survival than the non-irradiated control. Results from both studies indicate irradiation damages red blood cells affecting them in storage and after transfusion.

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INTRODUCTION

Irradiation of red blood cell (RBC) units has recently become a topic of special concern as the result of increasing reports of graft versus host disease (GVHD) in immunocompetent blood transfusion recipients.[1-12] The majority of medical facilities do not have the capability to irradiate blood products, requiring irradiated units to be stored.[13] Guidelines and standards for stocking irradiated blood products are being developed by regulating agencies. Few studies have been performed evaluating red cell survival *in vivo* following irradiation. More information is required before regulatory standards can be published.

Blood product irradiation has been widely accepted as the most effective prevention of GVHD in bone marrow transplant recipients and patients with severe combined and cellular immunodeficiencies. The medical facilities treating these patients usually have the capability to irradiate blood products prior to transfusion without the need for any significant storage period. Increasing numbers of reported cases of GVHD in nonimmunocompromised recipients, and the recommendation by the American Association of Blood Banks (AABB) that all cellular blood components from first degree family members should be irradiated with at least 1500 rads[14] necessitates the availability of irradiated blood products to all facilities that provide transfusions, as well as the tertiary treatment facilities supporting bone marrow transplantations and severely immunocompromised patients.[15] The additional risk of GVHD needs to be

addressed when family member donors are considered. It may not be cost effective for the facility that deals with only an occasional directed donor to own an irradiator.[16]

Normal Red Blood Cell Physiology

The membrane structure and metabolism of the red blood cell are closely related to the function and survival of the RBC in the circulation.[17] The RBC's primary function is to transport oxygen and carbon dioxide. The RBC membrane is composed of a lipid bilayer supported by a protein cytoskeleton.[18] The membrane is freely permeable to water and anions, but relatively impermeable to cations. Intracellular concentrations of sodium and potassium maintain the RBC volume and water balance; the red cell intracellular to extracellular ion ratios are 1:12 for sodium and 25:1 for potassium.[18] Adenosine triphosphate (ATP)-requiring cationic pumps actively transport sodium out of the cell and potassium into the cell to maintain these ratios. Any increase in membrane permeability or change in cationic transport may lead to a decrease in RBC survival.[18]

Nonfunctional RBCs are cleared from the circulation by the spleen; 1% is normally removed and replaced each day.[17] Red blood cells generate energy through anaerobic glucose metabolism; additional side pathways function as alternate methods of ATP production, to maintain 2,3-diphosphoglycerate (2,3-DPG) levels and maintain hemoglobin in the proper state.[18]

Potassium

Potassium, the major intracellular cation, is critical to the osmotic balance of cells. It functions in the body primarily for the transmission of normal muscle and nerve impulses, and is involved with cellular membrane potential. It also influences and is influenced by the acid base balance.[19] Normal serum concentrations of potassium are 3.5 to 5.3 mmol/L; the normal RBC intracellular concentration is 105 mmol/L.[19] This large difference in concentration is maintained by active transport of sodium and potassium across the cell membrane. Sodium is pumped out and potassium pumped into the cell.

The body's major regulation of potassium levels occurs in the kidneys. There it is filtered by the glomerulus, reabsorbed in the proximal tubules and reexcreted by the distal tubules of the nephron. Because there is no threshold level, the excretion continues even with decreased serum potassium concentrations. A constant dietary intake is needed to maintain the body's potassium level. Acid base changes significantly affect intracellular and extracellular potassium concentration. Potassium moves into the cells during metabolic alkalosis and out of the cells in acidosis.[20] This is caused by the movement of hydrogen ions and the body's need to balance ionic charges as ions move.

Increased serum potassium levels most commonly result from renal insufficiency or failure; renal dialysis is often required to remove

accumulated plasma potassium. Increased extracellular potassium causes changes in muscle irritability, respiration and heart function with electrocardiographic changes.[19] Symptoms may be seen with serum potassium concentrations greater than 7.5 mmol/L with concentrations greater than 10 mmol/L possibly being fatal.[19] During blood transfusions it is possible for large quantities of plasma potassium to be suddenly put into the circulatory system and to upset the delicate balance and overwhelm the body's compensation mechanisms. In healthy patients there is less concern because their compensation mechanisms rapidly respond to the potassium levels in the volumes of blood usually transfused. Plasma potassium contained in small volume transfusions gets diluted to levels of insignificance, while larger volume transfusions, especially when rapidly transfused, may raise the extracellular concentration and sufficiently overwhelm the compensatory mechanisms to cause serious results. The situation is compounded when the patient has impaired renal function as a result of disease or age. Neonates less than four months old requiring blood transfusions are a special concern as they have a small blood volume and poorly developed compensation systems.

Effects of Storage on Red Blood Cell Physiology

Effects of RBCs stored in accepted anticoagulants and under refrigeration result in a red cell storage lesion; this lesion results from decreases in pH, plasma sodium, 2,3-DPG, and ATP and increases in plasma hemoglobin and plasma potassium.[21,22] These changes are attributed primarily to the

continued metabolism of the stored cells. Accumulation of by-products, utilization of nutrients and diffusion of ions across the membrane occur during storage; the active transport of ions across the membrane is inhibited by refrigeration temperatures.[21,23] These changes do not significantly affect the RBC or its benefit to recipients during the 35 day shelf life of a unit of blood. Adenosine triphosphate levels have been used as a rough indicator of RBC viability in some studies.[21] Levels of 2,3-DPG are needed for proper oxygen carrying capabilities; they drop following a few days of storage, but quickly return to normal once transfused. Fifty percent of normal 2,3-DPG is restored in 3 - 8 hours and 100% restoration occurs in 24 hours.[24] Buffer systems in the collected blood and in the anticoagulant solution maintain the pH of the unit of blood within a physiologic range for RBCs. Sodium and potassium ions diffuse across the red cell membrane; in the body this movement of ions is corrected by active transport back across the cell membrane by the sodium-potassium pump. Any conditions that either decrease the activity of the active transport mechanism or increase the leakage of potassium out of the cell result in increased extracellular potassium levels.[21,23] The rise in extracellular potassium in units stored at 4^{0} C for 35 days is the result of a nonfunctional sodium-potassium pump. Decreased levels of ATP result from the cold temperature inhibition of glycolysis and utilization. Once transfused, RBCs revive and the pump and glycolysis resume normal functioning; levels of sodium, potassium, and ATP return to normal.[23]

Human Leukocyte Antigen System

The major histocompatibility complex (MHC) is the gene cluster that controls the expression of the human leukocyte antigens (HLA) found on the surface membranes of all cells. These antigens are essential for immune recognition and regulation. The gene complex is made up of several very closely linked loci, that are inherited as a group with rare crossing over. This antigen system is very polymorphic, having many different possible genes at each locus. Because of this uniqueness of "self", the best chance for a compatible transplantation donor is a blood relative. There are three classes of HLA antigens, i.e., class I, II and III. Class I HLA antigens are found on all nucleated cells and platelets and specifically function as cell surface recognition molecules; they are determined by the A, B and C loci. Class II HLA antigens are found on B lymphocytes, macrophages, monocytes, activated lymphocytes and a few other body cells that frequently come into contact with foreign substances.[25] They function in inducing cooperation and interaction between immune system cells and are determined by the DP, DQ and DR loci. Class III antigens are soluble plasma proteins that function as components of the complement system; they are not a factor in transplantation.[25] Class I and II antigens of the HLA system along with antigens of the ABO blood group system are the primary antigens of concern with solid organ transplantation. The HLA system is rarely involved in blood transfusions, but is a factor with some cases of transfusion associated GVHD.[11,26]

Immunology of Blood Transfusion

A blood transfusion is in part the transplantation of living tissue. Transfused blood introduces foreign cells and proteins into the recipient's system which may trigger an immune response. Normal compatibility testing matches donor blood only for the major ABO and Rh antigens and any blood group antigens against which the recipient has antibodies directed; but the immune response may be a cellular response to antigens and cells present in the transfused blood. Not all blood recipients respond the same way; they may not respond at all. The immune response or non response to transfused white blood cells is the same as with red cells.

Lymphocytes are responsible for recognition of non-self antigens. Normal circulating lymphocytes are in a resting state, waiting for foreign antigens to activate them to proliferate and mature into effector cells.[25] Activated lymphocytes acquire new receptors, enlarge, become more metabolically active and undergo mitosis to produce enough cells specific for the offending antigen to mount an effective immune response. B cells become antibody producing cells and memory cells; T cells become accessory cells and cytotoxic cells. Foreign antigens must be properly presented to the lymphocytes by monocytes or macrophages that have self class II HLA antigens for the lymphocytes to recognize.[25]

The cytotoxic immune response to transplanted cells is an artificial

situation that uses a system designed for the recognition and destruction of virally infected cells. Foreign, transplanted cells do not need to be recognized with self MHC molecules in order for the lymphocytes to respond; foreign MHC molecules can independently evoke an immune response against the cells. T cells can cause graft rejection although the precise mechanism of recognition is unknown.[25]

Graft Versus Host Disease

Graft versus host disease has long been recognized as a serious complication with bone marrow transplantation and transfusion of severely immunocompromised patients. The disease results when transplanted or transfused white blood cells, primarily immunocompetent T lymphocytes, recognize the histocompatibility antigens in the host as different and consequently proliferate and reject the host.[10] Recent cases of GVHD have been reported in nonimmunocompromised patients undergoing major surgery such as open heart surgery or cholecystectomy. Review of Japanese reports reveals many cases of what has been referred to as postoperative erythroderma that is now increasingly felt to be transfusion associated GVHD.[1-12]

There are three basic factors necessary for the development of GVHD. The first factor is histoincompatibility of host and transfused or transplanted cells. The second factor is immunoincompetence of the host, meaning the host is incapable of eliminating the transfused or

transplanted cells. The final factor is the capability of the transfused or transplanted cells to proliferate and attack the host *in vivo*.[27-29] Normally any transferred lymphocytes are seen as foreign and rapidly attacked and destroyed by the recipient's immune system. In GVHD the viable lymphocytes transferred are not recognized, attacked, or destroyed for several possible reasons; but those lymphocytes do see the recipient as foreign with resulting engraftment, blast transformation, and progression of rejection. This lack of recognition and destruction can be the result of congenital or acquired cellular or combined immunodeficiency[30] or an absence of the host recognizing the cells as foreign as a result of HLA similarities.[4,6,8,11,31] Post surgical GVHD is a result of a combination of factors that stimulate donor lymphocytes and depress host immune response.

Risk Factors

The patients at risk for GVHD and those who should receive irradiated blood products are not firmly established; as therapies become more aggressive and immunosuppressive the list will probably lengthen. Well documented situations for irradiated products include bone marrow transplantation recipients (autologous,[32] syngeneic, or allogeneic), patients suffering from severe cellular or combined immunodeficiency, and infants receiving intrauterine transfusion and later exchange transfusions.[33] Protocols for bone marrow transplantation preconditioning and severe immunodeficiency conditions, whether congenital or acquired,

render the patients' immune system incapable of rejecting foreign cells. Introduction of viable lymphocytes during fetal life via an intrauterine transfusion appears to be a prerequisite for GVHD in most neonates, but the source of the cells causing the disease is the exchange transfusion donor.[34] Immaturity of the immune system and confusion of self and non-self induced by the intrauterine transfusions results in a state of immune tolerance.[26,33] Less widely accepted situations include exchange transfusions of all premature infants, where it is thought that the infant immune system is too immature to respond properly, and leukemia and lymphoma patients, especially during the period of chemotherapy induced bone marrow suppression.[35,36] Questionable situations include those instances when blood donors are primary family members, transfusions in support of aplastic anemia and acquired immunodeficiency syndrome, and the use of intense chemotherapy[37] for patients with nonhematologic malignancies except neuroblastoma. Acquired immunodeficiency syndrome has a theoretical risk, but no cases have been documented.[26,33]

Congenital abnormalities known to be associated with GVHD are Di-George syndrome, severe combined immunodeficiency syndrome (SCIDS), Wiskott-Aldrich disease, and thymic aplasia. Acquired conditions known to be immunosuppressive and associated with GVHD are infections, high dose radiation, chemotherapy, immunosuppressive therapy and some malignancies.[26,30,33] The incidence of GVHD is likely to increase as more aggressive treatment protocols are developed for patients with malignant

disease, and granulocyte and platelet transfusions are more widely used.[37]

Situations becoming more associated with GVHD, include surgical stress and acute blood loss greater than 500 ml.[1,38] Transfusion of plasma, or products containing plasma is increasingly associated with immunosuppression[39] and cancer recurrence; soluble HLA class I antigens are thought to be the primary cause.[40] Surgically induced immunosuppression, a fairly well known phenomenon, has recently received more attention. Several factors with complex interrelationships appear to be associated with a wide range of immunosuppressive effects. Basic factors include anesthesia,[2,38] cardiopulmonary bypass and blood transfusion,[2] especially during surgery.[7,39,41] Additional factors that influence immunosuppression include the type of surgery,[2,7] medications,[8] age of the blood transfused[8,9,31] and the HLA relationship of the donor recipient pair.[6-8,11] Surgery induces significant temporary depletion of all types of circulating lymphocytes. Lymphocyte numbers recover in 24 to 48 hours, but they respond poorly to stimulation.[39] Steroid therapy alone causes a T-cell lymphopenia[8] and this can be compounded when combined with other factors.

Cardiac surgery appears to be the most immunosuppressive type of surgery,[7] most likely the result of the combined effects of the cardiopulmonary bypass, blood transfusions, and duration of the surgical procedure. Immunosuppression related to cardiopulmonary bypass appears to be caused by decreased complement components because of activation, consumption and

hemodilution; decreased immunoglobulins from hemodilution; and activation and proliferation of lymphocytes as the result of gram negative bacteria and endotoxin resulting from surgically induced stasis and ischemic injury of the bowel or primary or reactivated viral infections.[2,7]

Blood loss and blood transfusion independently affect the immune system. In one study mitogen response was significantly decreased in patients who received blood during surgery as compared to those who received blood only after surgery. The *in vivo* hypersensitivity skin test response was still declining 1 week post surgery and remained depressed for 2 - 3 weeks.[38,39,41] It has been questioned whether there is correlation between laboratory data and clinical findings with the test methods used.[41]

Immunocompetent recipients are increasingly associated with posttransfusion GVHD when the donor is HLA homozygous for a haplotype in common with a heterozygote recipient.[11,26] This situation specifically has been seen with family member donor recipient pairs or in populations with smaller genetic pools such as Japan or Israel.[6,26,42] Most recently reported cases of GVHD in immunocompetent individuals follow this pattern and include a match with one of the common extended haplotypes for a particular population.[6] In the Japanese population this haplotype match is felt to occur in one in every 3000 random donor transfusions.[31] The possibility of a random donor recipient HLA match in the United States has been considered to be very remote; however, there are communities within

the United States where this random match would be much more likely.[6] There are other factors that are felt to have influenced the more recently reported cases of GVHD in immunocompetent patients in Japan, and even cases in Israel and the United States. Most included the use of fresh blood with the partial HLA match from a related donor[6] in cases of cardiac surgery using cardiopulmonary bypass. All of these individually influence the immune status of the recipient and the combined effect is sure to be even more pronounced.

Most authors have stated that 1×10^7 lymphocytes per kilogram body weight[26,27,30,33,42,43] are needed to engraft; cases have been reported where the implicated blood products were estimated to contain as few as 1×10^4 lymphocytes/kg body weight.[30] It is likely that a balance between the degree of immunodeficiency, the dose of viable lymphocytes, and the degree of HLA match is required for GVHD to occur. Circumstances of each case must be evaluated individually. If multiple transfusions are given, there is a cumulative effect of viable immunocompetent lymphocytes sufficient to initiate a GVHD reaction.[44] Most non-frozen blood products contain numbers of viable lymphocytes well within the implicated range (Table 1); frozen cellular products contain preserved lymphocytes because of the cryoprotectants.[30] Products that have not been implicated in transfusion associated GVHD are frozen or processed plasma products and frozen and deglycerolized RBCs.[30] Transplanted organs may also be the source of lymphocytes.[26]

Table 1

Absolute Lymphocyte Counts in Blood Products[45]

Component	Lymphocytes per Unit
Red Blood Cells/Whole Blood	$1.0 - 2.0 \times 10^9$
Washed Red Blood Cells	$1.0 - 2.0 \times 10^8$
Filtered Red Blood Cells	$<1.0 \times 10^7$
Frozen and Deglycerolized RBCs	5.0×10^7
Random Platelet Unit	4.0×10^7
Pheresis Platelet Unit	3.0×10^8
Granulocyte Concentrate	1.0×10^{10}
Fresh Single Donor Plasma	1.5×10^5
Fresh Frozen Plasma	0
Cryoprecipitate	0

Diagnosis

Diagnosis of GVHD is complex and requires a degree of suspicion.[30] Clinical signs and symptoms include fever; acute erythematous rash, often progressing to desquamation; gastrointestinal symptoms of anorexia, nausea, vomiting, and profuse diarrhea; liver dysfunction demonstrated by abnormal laboratory values; and pancytopenia resulting from bone marrow involvement found only in cases of transfusion associated GVHD.[2,26,33,43,46] Confirmation methods are usually histologic examination of skin, liver, gastrointestinal, or bone marrow tissues; or, demonstration of circulating lymphocytes that are not the patient's HLA type but that of the blood or tissue donor.[35] HLA typing or karyotype analysis of the circulating lymphocytes or other tissues (see above) can be difficult because of the small numbers of circulating lymphocytes at the time of diagnosis.[26,33] Characteristic histologic findings of skin and gastrointestinal tract biopsy include edema, atrophy, degeneration of basal cell layers of epidermis, lymphocytic infiltration, hyperkeratosis, and mummified cells. Usual findings in bone marrow biopsy are hypoplasia to aplasia, lymphocytic infiltrate and evidence of erythrophagocytosis.[26,33]

Clinical onset in posttransfusion cases is usually very rapid, occurring within 4 - 30 days of the transfusion with nearly all cases very severe (grade IV). In contrast, the usual course in post transplantation cases is onset at 35 to 70 days, with severity varying from mild to severe (grades I - IV). Transfusion associated GVHD is nearly always acute,

while posttransplantation GVHD is variable between chronic and acute.[26] Cause of death in most cases of acute GVHD is overwhelming infection and sepsis.[30,33] Graft versus host disease can mimic many other common complications of the patients' primary diseases and their treatments, such as infections or drug reactions.[30,42] Timing of onset and similarity to other complications may make the association with the transfusion very difficult.[2]

Treatment

Treatment options for GVHD are limited and response varies greatly.[26,42] Chronic GVHD is more responsive to treatment than is acute, regardless of the cause. The usual therapies include corticosteroids, thalidomide, azathioprine, anti-thymocyte globulin, and cyclosporine; other therapies have been tried with limited success.[33] Transfusion associated GVHD has a much poorer prognosis and treatments have proven to be ineffective largely because of the bone marrow involvement. Most reports give greater than 90% fatality rates for transfusion associated GVHD.[26,30,33] Most posttransfusion cases go untreated because they remain undiagnosed until after death.[7] It is possible that only fatal cases of GVHD are recognized, this is especially true in the non-immunocompromised patients where the suspicion of the disease is especially low.[33] When GVHD is diagnosed, it is usually after it is well established, further limiting the effectiveness of treatment.[30] Assessing real incidence and the full range of the condition and fatality rates is a problem because of

underreporting.

Prevention

Prevention is much more promising than treatment.[30,43] The most efficient method used to prevent transfusion associated GVHD is to irradiate with 1500 to 3000 rads of gamma radiation all blood products for patients at risk.[47,48] Theoretically, the removal of the lymphocytes from blood products prior to transfusion would also prevent GVHD, but this is still unproven.[10,12,33,42] The use of ultraviolet irradiation is another method being studied to prevent transfusion associated graft versus host disease. Ultraviolet radiation also reduces sensitization to HLA antigens and refractoriness to platelet transfusions.[42,49] Although the use of fresh blood is considered an additional risk factor to transfusion associated graft versus host disease,[8,9,11,31] storage of blood components does not make the lymphocytes incapable of inducing a graft versus host response. Even though the number of viable cells decreases over time,[30,33] a significant degree of mitotic activity was found in mononuclear cells cultured from stored blood[27] as long as 22 days after storage.[50,51]

Gamma irradiation affects the DNA in the lymphocytes and prevents them from engrafting and proliferating.[33] Studies have shown that as little as 500 rads destroys the mitotic capability as measured by the mixed lymphocyte culture (MLC) while a dose of 1500 rads is needed to significantly reduce the mitogen response.[26] The MLC reactivity is

considered more representative of the ability of cells to induce graft versus host disease.[1,33,47] The currently accepted radiation dose is 1500 to 3000 rads; 1500 rads is most often used.[33] This dose allows a margin of safety in the event of uneven irradiation of a product or minor mechanical deviations.[33]

Many issues remain unresolved regarding the effects of irradiation of blood products. Damage that occurs to components is dose dependent, so it is important to use the minimal dose necessary to prevent GVHD.[33] Specific results vary with the study, but *in vitro* indicators of platelet function support that platelet concentrates irradiated with less than 5000 rads are acceptable for transfusion through 5 days storage.[26,33,52-56] Red blood cells are acceptable for transfusion when irradiated with the recommended dosage,[26,33,52,54,57] but effects of storage after irradiation is not well studied and storage is not recommended. The clinical significance of the observed elevation of plasma potassium levels in irradiated RBC units has been debated by some researchers.[13,14,26,58] Some authors recommend washing the units prior to transfusion[59] while others argue that it is unnecessary.[10,60] Results of granulocyte studies vary, but the overall consensus is that doses within the recommended range are acceptable.[27,33,52]

The Food and Drug Administration (FDA) advises that irradiated products are not licensed, complicating logistics of acquiring needed irradiated products for those facilities not having their own irradiators.

Irradiated products should be permanently labeled as irradiated, but may be used for patients in which irradiated blood products are not required.[14,26,33] The FDA additionally cautions that there may be problems with potassium levels in irradiated red cell products, especially when stored. Further information and guidance is anticipated pending review and release by the FDA.[14]

There have been several concerns raised of theoretically possible complications from the use of irradiated blood components. One of these concerns is whether irradiation could cause mutations leading to future malignancies in the blood recipient.[15,61] There is no risk of mutations as the radiation dose given is lethal by a safe margin, far exceeding the DNA repair mechanism.[33] Whether irradiation could release viruses contained in white blood cells has not yet been scientifically resolved; the information is limited to no reports of increase in infections in patients receiving irradiated blood products. Another question is whether irradiation could be a method to destroy viral RNA or DNA,[15] but data have indicated that the doses necessary are 100 to 1000 times that used for blood product irradiation, and that those doses are harmful to all cellular elements of blood products.[48] Irradiated blood products are not radioactive[16,33] and there is no threat to recipients or staff handling the units. The only people at risk of radiation exposure are the staff members who operate the irradiator.

Mechanics of Irradiation

The irradiation of blood products is accomplished either by using a specially designed blood product irradiator (usually with a cesium 137 gamma source) or by using a cobalt 60 source available in the nuclear medicine department.[16,33] The blood product irradiator is preferred because a more even and consistent dose is given and the blood bank retains control of the blood products; the irradiator is operated and controlled by the transfusion medicine department and not dependent on another department in the hospital.[26] A recalibration of dose is required for each use with the nuclear medicine cobalt 60 source.[48] Irradiators are expensive to purchase and operate. The gamma radiation hazards of the cesium source require regulation of installation, operation, and personnel.[16] Although the cesium is well shielded and poses little radiation hazard, irradiators are still regulated by the Nuclear Regulatory Commission and require special licenses. Quality control of irradiation equipment is important. Time of irradiation, decay of the cesium source, and effectiveness of MLC responses should be checked periodically.[62] The appropriate time period for these checks has not yet been established by regulatory agencies; 6 month or 1 year intervals seems most widely accepted.[14] Safety checks such as leakage tests, and personnel monitoring are usually performed by the radiation safety department.[16]

Blood irradiators are simple to operate. Blood products are placed into the instrument and the irradiator moves the blood products into the

radiation field. The radiation dose is controlled by the time exposed in the radiation field.

Red Blood Cell Survival Studies

The goal of RBC preservation is to provide functional blood components for transfusion purposes. Red blood cell viability is a measure of *in vivo* cell survival following transfusion and is defined by regulatory agencies as at least 70% survival of transfused RBCs 24 hours after the transfusion.[24] *In vivo* cell survival is the best indicator of any adverse effects as the result of blood product manipulations; all factors in cell viability while in the circulation are involved. The usual method to determine *in vivo* RBC survival is the tracking of infused chromium 51-labeled RBCs and their disappearance from the circulation. The time period monitored extends only to 24 hours when evaluating RBC storage conditions; it has been found that those cells surviving 24 hours have a normal life span.[63] Regulatory agencies have adopted 24 hour RBC survival as the standard to evaluate new preservatives and storage conditions.

The radioactivity of infused chromium 51-labeled RBCs at time zero is determined and called 100%; the radioactivity at 24 hours is determined and the percent survival calculated. Radioactivity at time zero is determined by measuring the blood volume using another isotope or is calculated from measurements taken immediately after infusion of the labeled blood. Key assumptions include: (1) that complete mixing of the

injected cells requires 2.5 to 3 minutes,[63,64] and (2) that the rate of cell loss in the first 20 minutes is steady.[63] Corrections must be made for the leaching of the isotope from the RBCs. There is considerable variation between individuals and techniques[64,65] making it difficult to standardize the test.

Fluorescent labeling of cells as a replacement for radioisotopes is a new development unavailable for routine use in humans. One fluorescent dye available, PKH26-GL (Zynaxis Cell Science, Malvern, PA), incorporates itself into the cell membrane, eliminating the radioisotope leaching problem.[66] Samples are analyzed on a flow cytometer; total cells are counted by forward and side light scatter and labeled cells are measured by their fluorescence at the dye's specific wavelength. The flow cytometer calculates the percentage of cells labeled from the fluorescent events and total RBC events. The dye is not immunogenic and does not alter the red cell function or life span when properly used.[66] Cell kinetic assumptions, similar to those for radioisotope labeling, are made.

Fundamentals of Flow Cytometry

Flow cytometry is the measurement of physical or chemical characteristics of particles while they pass single file in a fluid stream through a measurement device.[67] The basics of present flow cytometry uses measurements of light scattering and fluorescence using a laser light source.[68] Large numbers of cells are analyzed in a short period of time

increasing the accuracy and specificity of the method.

The flow cytometer consists of four basic subsystems. The fluidics subsystem carries the cells from the tube to the sensing region. The optical subsystem generates, isolates and collects cell-derived light. The electronic subsystem amplifies and digitizes the light signals. The data management subsystem translates the electronic signals into readable graphics and numbers.[58]

Cells in suspension are forced into the center of a stream of fluid; the design of the flow chamber causes the cells to pass one at a time through a quartz cuvette where they are exposed to a beam of focused monochromatic laser light. The intense light is scattered by the cells and excites fluorescent dyes, when present, causing them to emit specific wavelengths of light. Forward light scatter measurements detect light deflected over a small range of angles, indicating the size of the cells. Side scatter measurements detect light scattered at an angle of 90 degrees from the beam of light, indicating the granularity of the cell cytoplasm. Separate detectors measure light of specific wavelengths to detect the light emitted by different fluorescent dyes. The signals from each of the detectors are classified by the electronic subsystem into a scale of over 1000 channels.[58] The greater the number of channels separating different cell populations the more clear the differentiation of the populations.

Flow cytometry is used for whole blood and cell subset analysis, reticulocyte and platelet counting, leukemia phenotyping, drug monitoring, immunogenetics and DNA cell cycle analysis. Fluorochrome-conjugated antibodies specific to certain cell markers assist in the differentiation of cell populations.[58]

Purpose of the Project

The purpose of this project was to determine if stored irradiated blood has significantly increased levels of plasma potassium as compared to non-irradiated stored controls, and to determine at what storage time the effects become significant. The survival after transfusion of stored irradiated RBCs as compared to non-irradiated stored controls was determined using a dog model. It was expected that plasma potassium levels of irradiated units of RBC stored more than 7 days would be elevated, but red cell survival would be normal at 24 hours after transfusion.

METHODS

In Vitro Study

Packed RBCs were obtained from the blood donor center (Walter Reed Army Medical Center, Washington DC; National Naval Medical Center, Bethesda, MD) within 3 days of collection. All units used were fully tested and processed following standard Food and Drug Administration (FDA) and AABB requirements. All units were collected into an 800 ml collection bag, (Fenwal, Division of Travenol Laboratories, Deerfield IL) containing citrate phosphate dextrose adenine (CPDA-1) anticoagulant. Units were divided into two aliquots using a sterile docking device (Haemonetics SCD 312, Haemonetics Corporation, Braintree MA) and 600 ml transfer packs (Fenwal, Division of Travenol Laboratories) or integrally attached satellite bags. Aliquots were weighed and hematocrits performed. Aliquots were stored at standard monitored blood bank temperature conditions.

One aliquot of each unit was irradiated with 3000 rads using a blood irradiator (model 143, J.L. Sheppard Inc., San Fernando, CA) with a cesium 137 gamma source (Walter Reed Army Medical Center). The time of exposure was calculated using the following formulas and figures. The central dose rate (CDR), i.e., 831 rad/min, was provided by the manufacturer at installation in November 1989. The cesium decay chart provided by the manufacturer indicated 97% activity remaining as of January 1991. The CDR

as of January 1991 was calculated using the following formula.

$$CDR = 831 \times 0.97 = 806.07$$

The average dose rate (ADR) was calculated using the following formula.

$$ADR = 0.89 \times CDR = 0.89 \times 806.07 = 717.40$$

Time required for the dose was calculated by dividing the required dose by the ADR.

$$\text{TIME REQUIRED FOR DOSE} = 3000/717.40 = 4.18 \text{ min}$$

All aliquots were sampled at 1, 3, 5, 7, 10, 14, 21, 28 and 35 days following irradiation. Testing at day 0 was added to establish a baseline after results of days 1 and 3 from the first three units were reviewed. Aliquots were mixed by gentle inversion and 3-5 ml of blood was withdrawn through a sampling site coupler (Fenwal, Division of Travenol Laboratories) using an 18 gauge needle and syringe (Becton Dickinson Labware, Becton Dickinson and Co., Lincoln Park, NJ). Samples were placed into silicone separator vacuum tubes (Corvac, Sherwood Medical, St. Louis, MO) after removing the needle from the syringe and the stopper from the tube. All sampling was performed in a laminar flow hood to reduce contamination. Immediately following sampling and centrifugation at high

speed for 10 min in a table top centrifuge, plasma was separated from the cells. Plasma was diluted 1:10 with distilled water to bring the potassium values into analytic range. Potassium analysis was performed in triplicate immediately after dilution using a NOVA 13 electrolyte analyzer (NOVA Biomedical, Newton, MA). A sample of each aliquot collected on day 35 was cultured on chocolate agar (Difco Laboratories Inc., Detroit, MI) incubated for 3 days at 37^0 C to assure sterility.

Statistical Analysis

The mean of the triplicate results was used for all statistical manipulations. Total aliquot potassium values were calculated using the following formulas.

PLASMA VOLUME in L = ALIQUOT WEIGHT in g x (1 - ALIQUOT HEMATOCRIT)/1000

TOTAL ALIQUOT POTASSIUM = POTASSIUM VALUE in mmol/L x PLASMA VOLUME in L

Paired results of potassium values and total aliquot potassium results were graphed over time. Paired t test as performed on Minitab statistical computer software (Minitab Inc., State College, PA) was used to analyze the results of each of the sampling days; a p value less than 0.05 and a t statistic greater than two was considered significant.

In Vivo Study

Five foxhounds (Walter Reed Army Institute of Research, Division of Veterinary Medicine, Forest Glen Annex, Walter Reed Army Medical Center, Washington DC) were used for the *in vivo* portion of the project; each dog was treated identically in five successive procedures with the exception that the unit of blood collected from dog 1 was not irradiated. All dogs were male.

Collection of Units

Each animal was weighed and its blood volume calculated (8.8% of body weight).[69] Approximately 10% of each animal's blood volume was collected from the jugular vein as a unit of blood following standard blood donor center procedures using 800 ml collection bags containing CPDA-1 anticoagulant (Miles Inc, Cutter Biological, Elkhart, IN). Each unit was weighed and processed into packed RBC units having a hematocrit of 70-80% by centrifuging at 4300 RPM for 5 min in a refrigerated centrifuge (Sorval, DuPont, Raritan, NJ). Plasma was expressed into an attached satellite bag. Units from animals 2, 3, 4 and 5 were irradiated with 3000 rads of gamma radiation following the same procedure as with the units of human blood. All units were stored at standard blood bank temperature conditions for 14 days.

Labeling of Cells

All open manipulations were performed using sterile techniques in a

laminar flow hood. An aliquot of well mixed blood equal to 100 ml of whole blood was taken from the unit using a plasma transfer set (Fenwal Laboratories, Division of Travenol Laboratories Inc.) inserted into one of the ports on the unit. The volume of packed cells needed was calculated using the following formula.

HEMATOCRIT OF UNIT X ml BLOOD NEEDED = 0.45 X 100 ml

The volume of blood was measured using sterile 25 ml disposable pipets (Costar, Cambridge, MA). The total volume was brought to 100 ml with sterile sodium chloride irrigation, 0.9%, USP (Kendall McGaw Laboratories, Inc., Irvine, CA) and mixed well by inversion. Ten ml of the aliquot of blood was placed into each of 10 sterile 50 ml plastic conical centrifuge tubes (Becton Dickinson Labware, Becton Dickinson and Co.). The volume in each tube was brought up to 50 ml with sterile sodium chloride irrigation, 0.9%, USP (Kendall McGaw Laboratories, Inc.), mixed well by inversion and centrifuged at 2200 RPM for 7 min to remove (wash) the plasma proteins from the red cells. Supernatant was removed using a vacuum suction apparatus. The wash was repeated once.

Fluorescent dye PKH26 (Zynaxis Cell Science Inc.) was diluted to 10×10^{-6} M concentration using supplied diluent. This concentration was determined by previous experiments (Appendix A). The volume of dye used

was determined using the following formula.

ml OF DYE x SUPPLIED CONCENTRATION = NEEDED VOLUME x NEEDED CONCENTRATION

$$ml\ OF\ DYE \times 1x10^{-3}\ M = 100\ ml \times 10x10^{-6}\ M$$

Ten ml of the diluted dye was added to each tube, mixed by inversion and incubated 4 min at room temperature with constant gentle inversion. Incubation time was determined by previous experiment (Appendix A). At the end of the incubation period the staining action was stopped by bringing the volume of each tube to 50 ml with a 1%, vol/vol, solution of fetal bovine serum (Gibco Laboratories, Life Technologies, Inc., Grand Island, NY); the tubes were mixed by inversion and centrifuged at 2200 RPM for 7 min. The fetal bovine serum (Gibco Laboratories, Life Technologies, Inc.) was heat inactivated for 30 min at 56^0 C, diluted in sodium chloride irrigation, 0.9%, USP, (Kendall McGaw Laboratories, Inc.) and sterile filtered with a 0.22 um bottle top filter (Becton Dickinson and Co.). Two more fetal bovine serum solution washes were performed. All tubes were then washed twice with sterile sodium chloride irrigation, 0.9%, USP (Kendall McGaw Laboratories, Inc.) following the same procedure described previously. Blood was pooled and drawn into two 60 cc syringes (Becton Dickinson and Co.). Filled syringes were kept in the dark and on wet ice until reinfused. One ml samples of washed unlabeled blood and labeled blood were kept refrigerated and in the dark for analysis on the

flow cytometer.

Reinfusion

All animals were calmed with acepromazine maleate injection (TechAmerica, Fermenta Animal Health Co., Kansas City, MO) at a dosage of 0.5 mg/lb body weight given approximately 1 hour prior to the procedure. A 16 gauge 2.5 cm intravenous catheter (Deseret Medical Inc., Becton Dickinson and Co., Sandy, UT) was placed in the cephalic vein of the front leg and a sodium chloride injection, 0.9%, USP (Abbott Laboratories, North Chicago, IL) was started at a rate of 3 drops/min to keep the line open. An 18 gauge 2.5 cm intravenous catheter (Deseret Medical Inc.) with a heparin lock (Burron Medical Inc., Bethlehem, PA) was placed in the saphenous vein of the rear leg on the same side of the animal for collection of the blood specimens. Blood for infusion was attached to the intravenous line with a three way stopcock (ABCO, Inc., Milwaukee, WI) and was infused at a rate of 0.5 ml/min/kg using an infusion pump (Harvard Apparatus, South Natick, MA). Blood samples were drawn prior to the reinfusion and at 3, 5, 7.5, 10, 12.5, 15, and 60 minutes following completion of the blood infusion. Blood specimens were drawn from the heparin lock using an 18 gauge needle and 3 ml syringe (Becton Dickinson and Co.); the line was cleared with 2 ml of blood prior to collection of each sample. Each blood sample was immediately placed into a 3 ml sodium heparin anticoagulated vacutainer tube (Becton Dickinson Vacutainer Systems, Rutherford, NJ). The heparin lock was filled with heparin solution after each sample was drawn. A blood sample was drawn from the cephalic vein 24 hours and 1 week after

the completion of the blood infusion. Blood specimens were refrigerated and protected from light until analysis. The intravenous catheter used for the blood infusion was removed after the 15 min specimen.

Hematocrits were performed on all units prior to washing, the washed unlabeled blood, the labeled blood ready to infuse, the preinfusion dog sample and the 1 hour post-infusion dog sample. Flow cytometer analysis was performed on samples from the washed unlabeled unit, the labeled blood, the preinfusion dog blood specimen and each of the timed post-infusion dog blood specimens.

Flow Cytometry Analysis

Approximately 0.5 ml of each well-mixed blood sample was washed twice by placing blood in a 12 x 75 mm test tube (Becton Dickinson and Co.) and filling with sodium chloride, 0.9%, (Baxter Healthcare Corporation, Scientific Products Division, McGaw Park, IL), centrifuging for 60 sec in a serofuge (Dade, American Scientific Products, American Hospital Supply Corp, McGaw Park, IL) and removing the saline. Five ul of washed blood was placed in 1 ml of phosphate buffered saline (American Scientific Products) and mixed for analysis. Flow cytometry analysis was performed on a Becton Dickinson FACScan (Becton Dickinson Immunocytochemistry Systems, Mountain View, CA) with the FSC (forward light scatter detector) and SSC (side light scatter detector) changed to log and the flow rate changed to low. The flow cytometer was calibrated by the Walter Reed Army Medical Center Diagnostic Immunology staff in accordance with

manufacturer's recommendations prior to each day's use. All analysis were performed on the same instrument. The excitation wavelength for the dye was 488 nm and the emission wavelength was 570 nm. This emission was in the orange range and detected by the FL2 detector of the FACScan (Becton Dickinson Immunocytochemistry Systems).

Statistical Analysis

Ten thousand cells were analyzed for each sample and each specimen was analyzed in triplicate. Using Consort 30 data analysis software (Becton Dickinson Immunocytochemistry Systems) graphs were generated for each sample demonstrating forward light scatter versus side light scatter and fluorescence emission on the FL2 detector. Labeled and unlabeled RBC populations were graphically separated and the number of labeled cells quantified on the FL2 channel. Linear and logarithmic regression analysis was performed using Minitab computer statistical software (Minitab Inc.) on the timed samples starting from the point at which the labeled cell population peaked and ending with the 15 minute specimen to establish the 100% red cell point. Each dog's RBC mass was estimated using the following formulas.

$$\text{BLOOD VOLUME in ml} = \frac{\text{WEIGHT in Kg} \times 0.088 \times 1000}{1.052}$$

$$\text{RED CELL MASS} = \text{BLOOD VOLUME} \times \text{HEMATOCRIT} \times 0.90$$

The 24 hour red cell survivals were calculated using the following formula.

$$\text{24 HOUR RED CELL SURVIVAL} = \frac{\text{PERCENT RBCs LABELED AT 24 HOURS}}{\text{PERCENT RBCs LABELED AT 0 HOURS}} \times 100$$

The percentage of labeled RBCs expected to survive after one week were calculated using the following formula.

$$\text{LABELED RBCs AT ONE WEEK} = \text{PERCENT RBCs LABELED AT 24 HR} - (\text{PERCENT RBCs LABELED AT 24 HR} \times 0.01 \times 6)$$

RESULTS

In Vitro Study

Potassium levels in irradiated aliquots rose sharply as compared to the non-irradiated controls (Table 2); this elevation appeared on day 1 and remained throughout the 35 day storage period. The potassium levels of the irradiated aliquots plateaued at approximately 2 weeks, while the non-irradiated aliquots continued a steady rise through the end of the testing period (Fig. 1). All irradiated aliquots displayed similar elevated potassium levels at each sampling point. The potassium levels of the irradiated aliquots remained two times the level of the non-irradiated aliquots throughout the first 2 weeks. The mean difference between the irradiated and non-irradiated pairs was statistically significant throughout the storage period; it peaked at 7 days and began to decline at 21 days. The mean difference was 0.1 mmol/L on day zero rising to 18.8 mmol/L on day 1, peaking on day 7 at 36.7 mmol/L and gradually declining to 21.7 mmol/L by day 35 (Table 3).

Total potassium content of each aliquot was calculated to illustrate unit to unit variation and to relate the results to clinical significance (Table 4). A rapid rise after irradiation was again displayed; total potassium content of the irradiated aliquots was about two times that of the non-irradiated aliquots (Fig. 2). The mean difference of the total potassium content between the irradiated and non-irradiated pairs displayed the same pattern as that of the potassium values (Table 5).

Table 2

Potassium values in mmol/L of irradiated versus non-irradiated aliquot pairs of human packed RBCs collected in CPDA-1 anticoagulant at various storage points after exposure to 3000 rads of gamma radiation.

Day	*Treatment	Unit Number 1	2	3	4	5	6	7	8	9	10	Mean
0	I				11.3	14.8	11.6	13.5	11.4	16.6	11.5	13.0
	N				11.5	14.1	12.5	12.9	11.5	16.3	11.5	12.9
1	I	46.8	51.7	45.2	28.1	28.1	28.4	35.4	31.0	41.3	31.6	36.8
	N	28.2	21.7	19.0	13.7	16.4	14.6	16.5	14.3	20.8	14.6	18.0
3	I	58.1	66.9	59.7	47.5	46.8	48.0	59.3	51.5	58.4	47.8	54.4
	N	32.8	29.0	24.8	20.4	22.4	21.8	24.0	19.8	27.4	19.8	24.2
5	I	67.7	77.7	69.8	52.7	51.9	54.9	66.5	60.5	68.5	59.5	63.0
	N	37.2	35.1	31.3	22.9	24.6	25.1	27.4	24.0	33.5	24.0	28.5
7	I	78.2	82.4	76.9	57.1	56.2	59.6	71.0	65.8	73.7	65.0	68.6
	N	37.8	40.0	36.5	25.5	26.7	28.3	31.0	27.5	38.4	27.1	31.9
10	I	83.5	92.5	84.6	63.9	64.2	67.2	79.4	73.4	80.0	70.7	75.9
	N	47.9	43.5	49.4	30.3	32.5	38.1	37.8	34.3	45.9	33.4	39.3
14	I	90.0	99.7	90.5	75.3	68.8	73.3	83.5	78.6	85.5	75.6	82.1
	N	56.0	57.5	54.5	36.7	37.1	40.9	42.9	40.0	52.8	38.0	45.6
21	I	86.3	102.5	88.3	76.0	75.5	78.0	91.4	84.6	90.4	81.0	85.4
	N	60.0	61.9	60.9	45.6	45.3	50.8	53.7	44.2	63.0	47.3	53.3
28	I	90.0	100.4	90.7	76.2	77.7	80.4	86.4	87.9	91.1	81.6	86.3
	N	66.9	70.8	67.9	51.3	51.7	57.4	61.5	57.4	70.8	52.9	60.9
35	I	96.3	106.0	96.1	77.6	81.3	82.4	97.9	90.6	92.5	80.8	90.2
	N	75.2	82.9	77.4	57.1	57.3	65.0	69.4	64.7	76.3	59.0	68.5

*I - Irradiated
N - Non-irradiated

FIGURE 1. Mean potassium values in mmol/L of irradiated versus non-irradiated aliquot pairs of CPDA-1 anticoagulated human packed RBCs over 35 days storage N=10. Irradiated aliquots were exposed to 3000 rads of gamma radiation prior to storage.

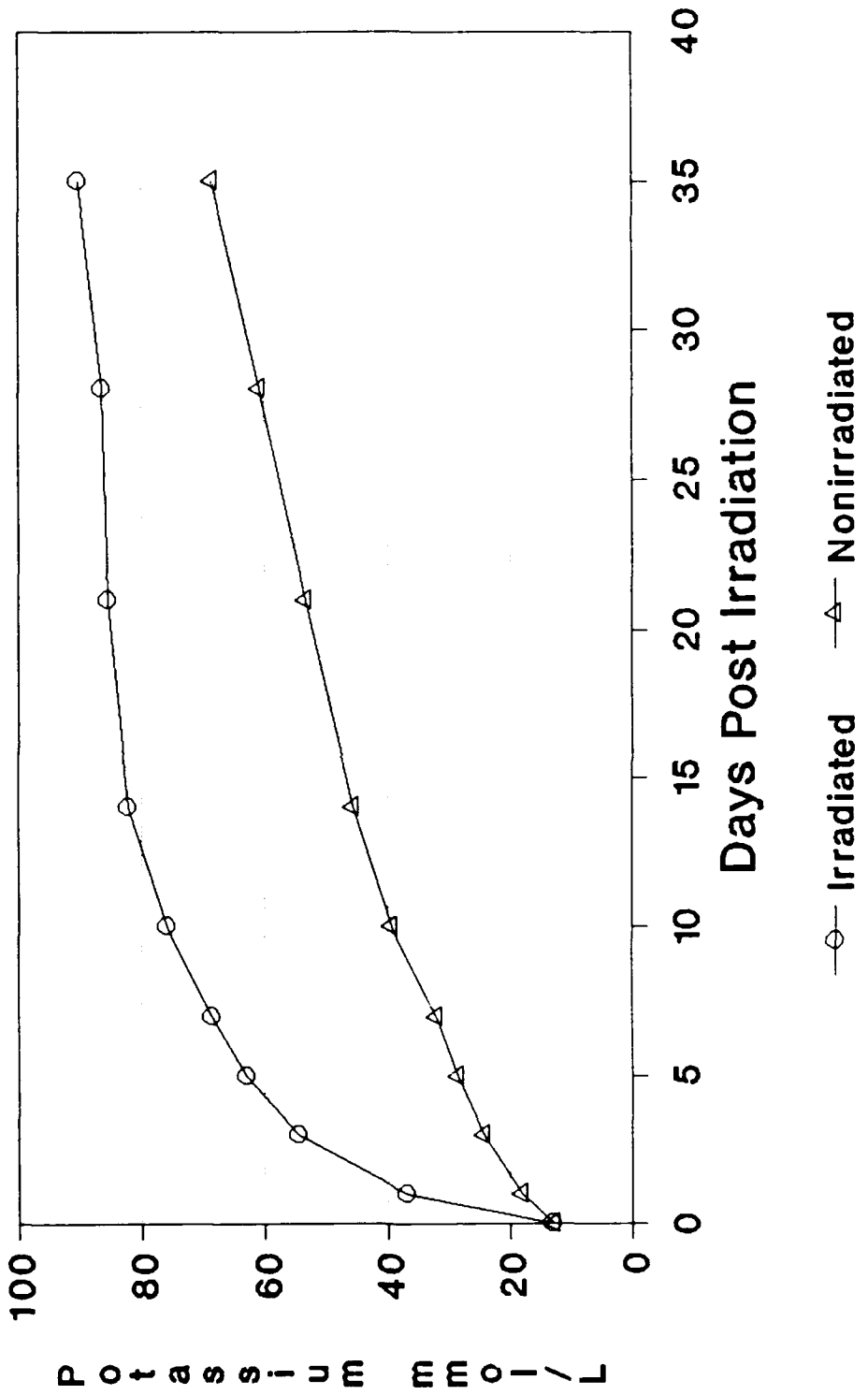

Potassium mol/L
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
Days Post Irradiation
Irradiated
Nonirradiated

Table 3

Mean potassium values in mmol/L of irradiated versus non-irradiated aliquot pairs of human packed RBCs collected in CPDA-1 anticoagulant at various storage points after exposure to 3000 rads of gamma radiation.

	Days Post Irradiation									
Treatment	0	1	3	5	7	10	14	21	28	35
Irradiated	13.0	36.8	54.4	63.0	68.6	75.9	82.1	85.4	86.3	90.2
Non-Irradiated	12.9	18.0	24.2	28.5	31.9	39.3	45.6	53.3	60.9	68.5
Difference	0.1	18.8	30.2	34.5	36.7	36.6	36.5	32.1	25.4	21.7
Number	7	10	10	10	10	10	10	10	10	10

Table 4

Total potassium content in mmol of each aliquot of irradiated versus non-irradiated human packed RBCs collected in CPDA-1 anticoagulant at various storage points after exposure to 3000 rads of gamma radiation.

Day	*Treatment	Unit Number 1	2	3	4	5	6	7	8	9	10	Mean
0	I					0.8	0.8	0.7	0.6	0.5	0.8	0.7	0.7
	N					0.8	0.8	0.8	0.6	0.6	0.8	0.7	0.7
1	I	2.7	2.2	2.5	2.0	1.5	1.6	1.7	1.5	2.1	2.0	2.0
	N	1.5	1.0	1.0	1.0	0.9	0.9	0.7	0.7	1.0	0.9	1.0
3	I	3.3	2.9	3.3	3.4	2.6	2.7	2.8	2.4	3.0	3.1	3.0
	N	1.8	1.3	1.3	1.4	1.2	1.3	1.1	0.9	1.4	1.2	1.3
5	I	3.8	3.4	3.9	3.7	2.8	3.1	3.1	2.8	3.5	3.8	3.4
	N	2.0	1.6	1.7	1.6	1.3	1.5	1.2	1.1	1.7	1.4	1.5
7	I	4.4	3.6	4.3	4.1	3.1	3.4	3.3	3.1	3.8	4.2	3.7
	N	2.0	1.8	2.0	1.8	1.4	1.7	1.4	1.3	1.9	1.6	1.7
10	I	4.7	4.0	4.7	4.5	3.5	3.8	3.7	3.5	4.1	4.5	4.1
	N	2.6	2.0	2.7	2.1	1.8	2.3	1.7	1.6	2.3	2.0	2.1
14	I	5.1	4.3	5.1	5.4	3.8	4.1	3.9	3.7	4.4	4.8	4.5
	N	3.0	2.7	3.0	2.6	2.0	2.5	1.9	1.9	2.6	2.2	2.4
21	I	4.9	4.4	4.9	5.4	4.1	4.4	4.3	4.0	4.6	5.2	4.6
	N	3.2	2.9	3.3	3.2	2.5	3.1	2.4	2.1	3.1	2.8	2.9
28	I	5.2	4.3	5.1	5.4	4.3	4.5	4.0	4.1	4.7	5.2	4.7
	N	3.6	3.3	3.7	3.6	2.8	3.5	2.7	2.7	3.5	3.1	3.3
35	I	5.5	4.6	5.4	5.5	4.5	4.6	4.6	4.3	4.7	5.2	4.9
	N	4.0	3.8	4.2	4.0	3.1	4.0	3.1	3.1	3.8	3.4	3.7

*I - Irradiated
N - Non-irradiated

FIGURE 2. Mean total potassium content in mmol of irradiated versus non-irradiated aliquot pairs of CPDA-1 anticoagulated human packed RBCs over 35 days storage N=10. Irradiated aliquots were exposed to 3000 rads of gamma radiation prior to storage.

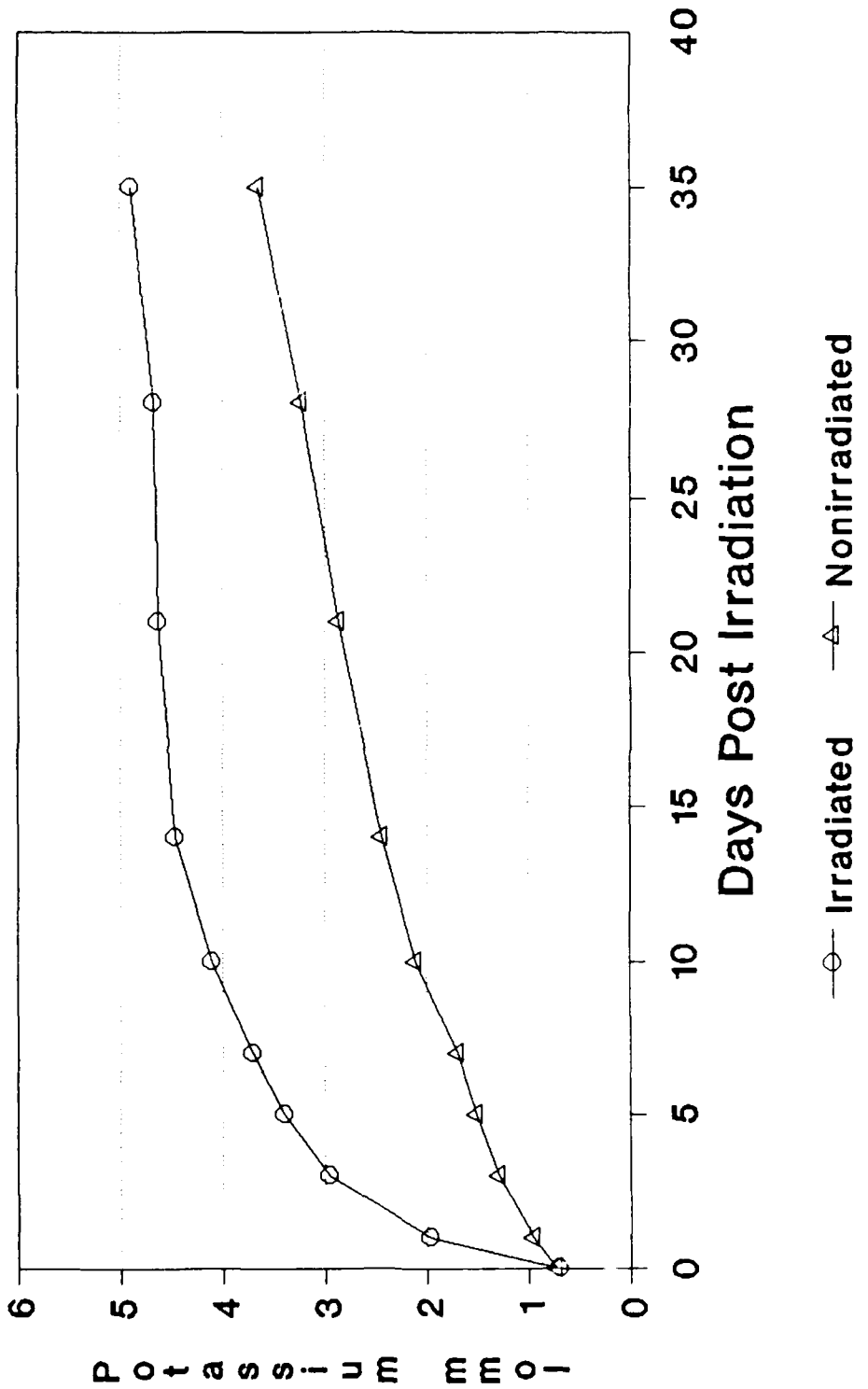

Potassium mol
0
1
2
3
4
5
6
0
5
10
15
20
25
30
35
40
Days Post Irradiation
Irradiated
Nonirradiated

Table 5

Mean total potassium content in mmol of each set of aliquots of irradiated versus non-irradiated human packed RBCs collected in CPDA-1 anticoagulant at various storage points after exposure to 3000 rads of gamma radiation.

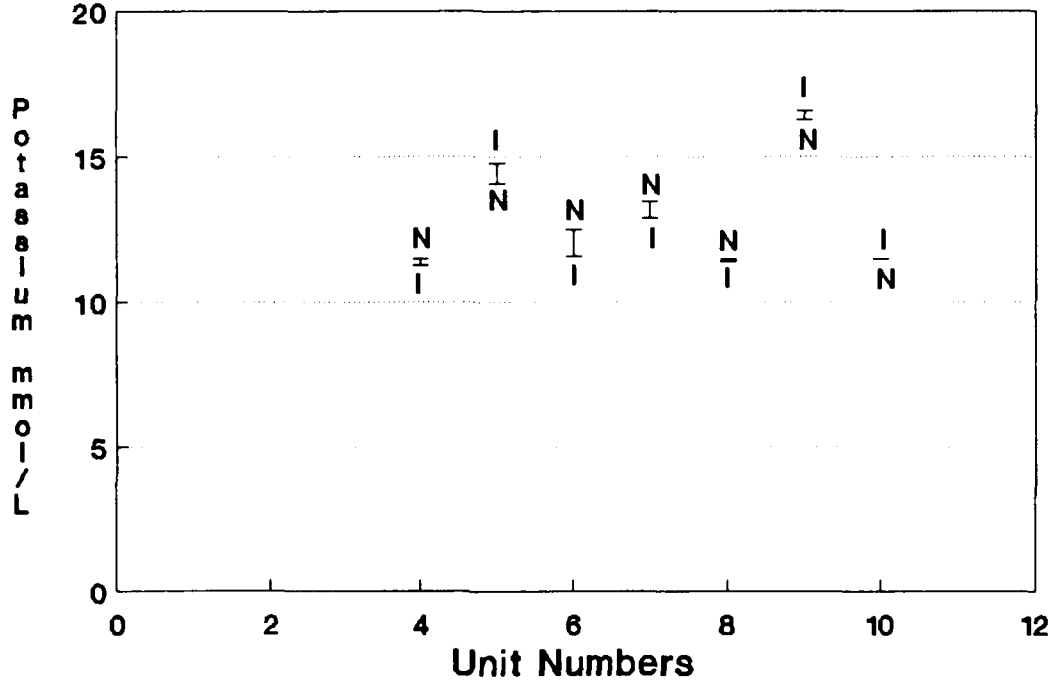
	Days Post Irradiation									
Treatment	0	1	3	5	7	10	14	21	28	35
Irradiated	0.70	1.98	2.95	3.39	3.72	4.10	4.46	4.62	4.68	4.89
Non-Irradiated	0.73	0.96	1.29	1.51	1.69	2.11	2.44	2.86	3.25	3.65
Difference	0.03	1.02	1.66	1.88	2.03	1.99	2.02	1.76	1.43	1.24
Number	7	10	10	10	10	10	10	10	10	10

A small insignificant difference was observed in potassium values between the irradiated and non-irradiated aliquots on day zero with a mean difference of 0.057, t statistic of 0.276 and p value of 0.79 (Fig. 3). An increase in potassium values of the irradiated aliquots on day 1 was observed with a mean difference of 18.78, t statistic of 10.551 and p value < 0.001 (Fig. 4). An increase in potassium values of the irradiated aliquots on day 7 was noted with a mean difference of 36.710, t statistic of 25.689 and p value < 0.001 (Fig. 5). The continued increase in potassium values of the irradiated aliquots was noted on day 14 with a mean difference of 36.440, t statistic of 31.550 and p value < 0.001 (Fig. 6). Increased potassium values of the irradiated aliquots to a mean difference of 32.130, t statistic of 18.112 and p value < 0.001 was observed on day 21 (Fig. 7); a mean difference of 24.470, t statistic of 24.680 and p value < 0.001 observed on day 28 (Fig. 8) and on day 35 a mean difference of 21.670, t statistic of 17.604 and p value < 0.001 (Fig. 9). The large difference of the irradiated versus non-irradiated aliquots for the 35 day storage period clearly exceeded the standard error (Fig. 10).

In Vivo Study

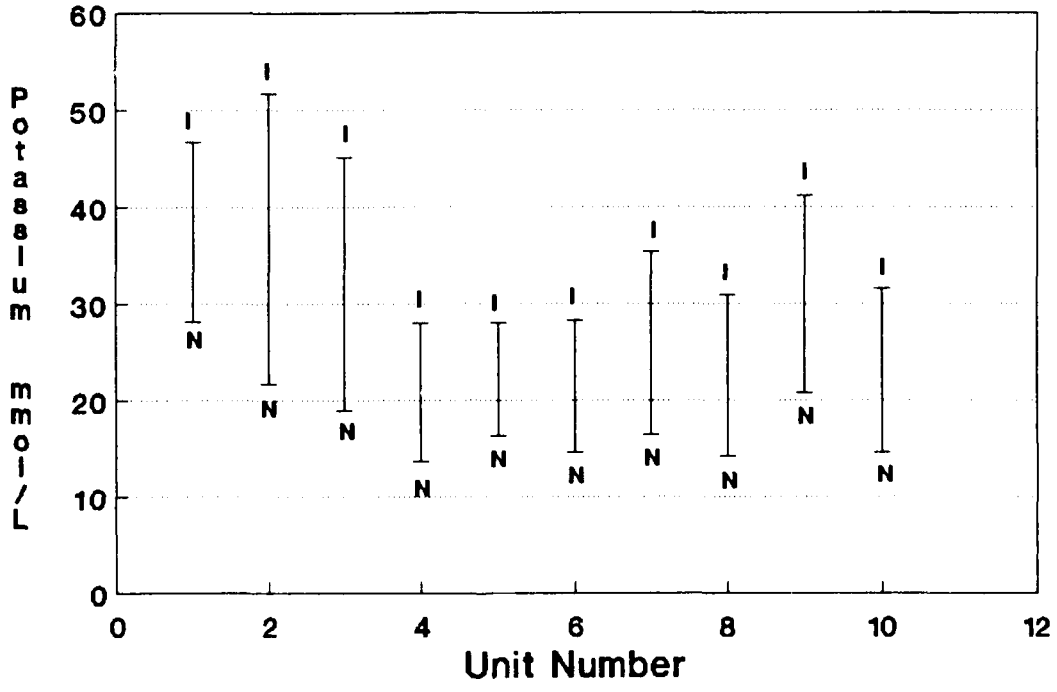
The weights of the five foxhounds used in the *in vivo* study ranged from 54 to 65 lbs, the calculated blood volumes ranged from 2049.4 ml to 2467.7 ml and the preinfusion hematocrits ranged from 29% to 39% (Table 6). The

FIGURE 3. Comparison of potassium values in mmol/L of individual aliquot pairs of irradiated (I) and non-irradiated (N) CPDA-1 anticoagulated human packed RBCs on the day of exposure to 3000 rads of gamma radiation and the statistics of the difference.



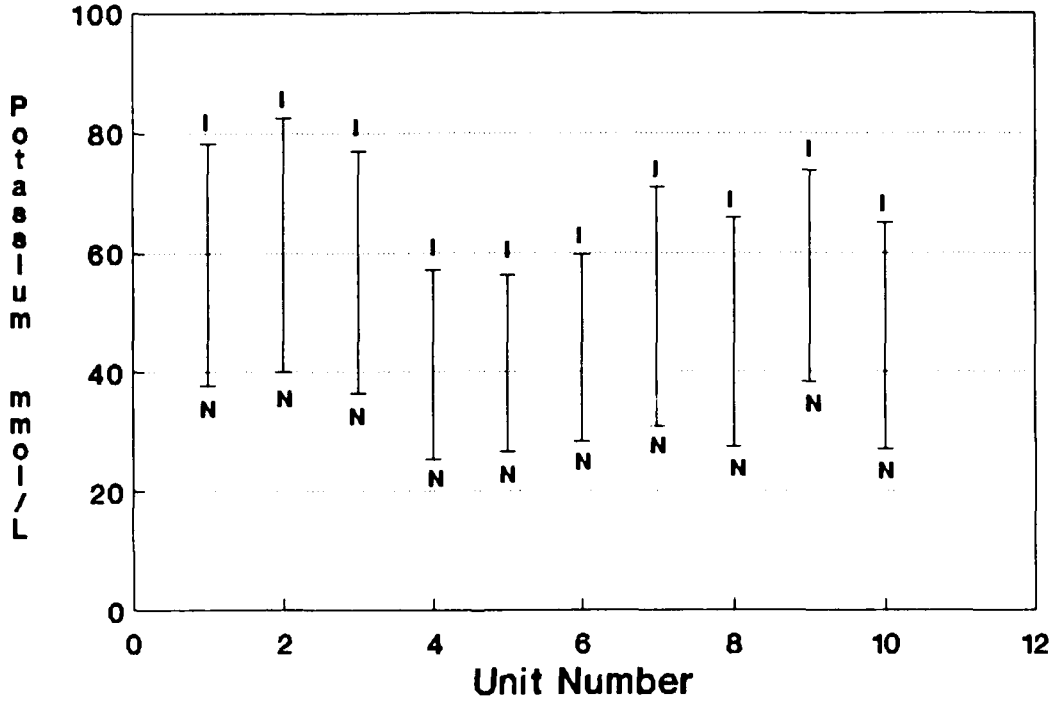
N	Mean	Standard Deviation	Standard Error	t Statistic	P Value
7	0.057	0.544	0.206	0.276	0.79

FIGURE 4. Comparison of potassium values in mmol/L of individual aliquot pairs of irradiated (I) and non-irradiated (N) CPDA-1 anticoagulated human packed RBCs on day 1 after exposure to 3000 rads of gamma radiation and the statistics of the difference.



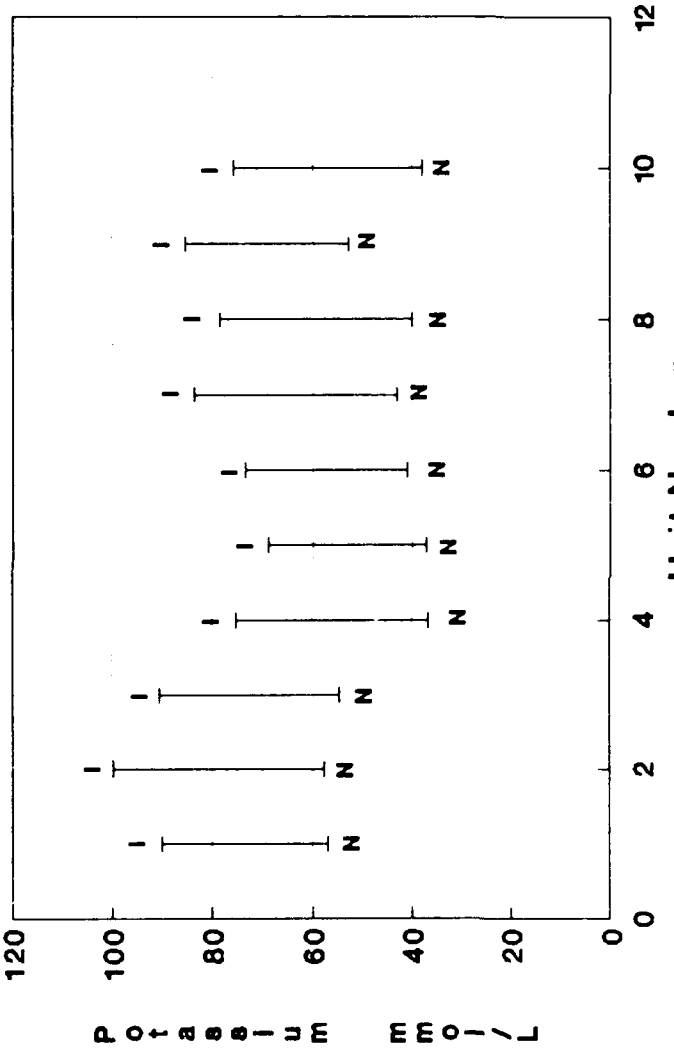
N	Mean	Standard Deviation	Standard Error	t Statistic	P Value
10	18.780	5.633	1.781	10.551	<0.001

FIGURE 5. Comparison of potassium values in mmol/L of individual aliquot pairs of irradiated (I) and non-irradiated (N) CPDA-1 anticoagulated human packed RBCs on day 7 after exposure to 3000 rads of gamma radiation and the statistics of the difference.



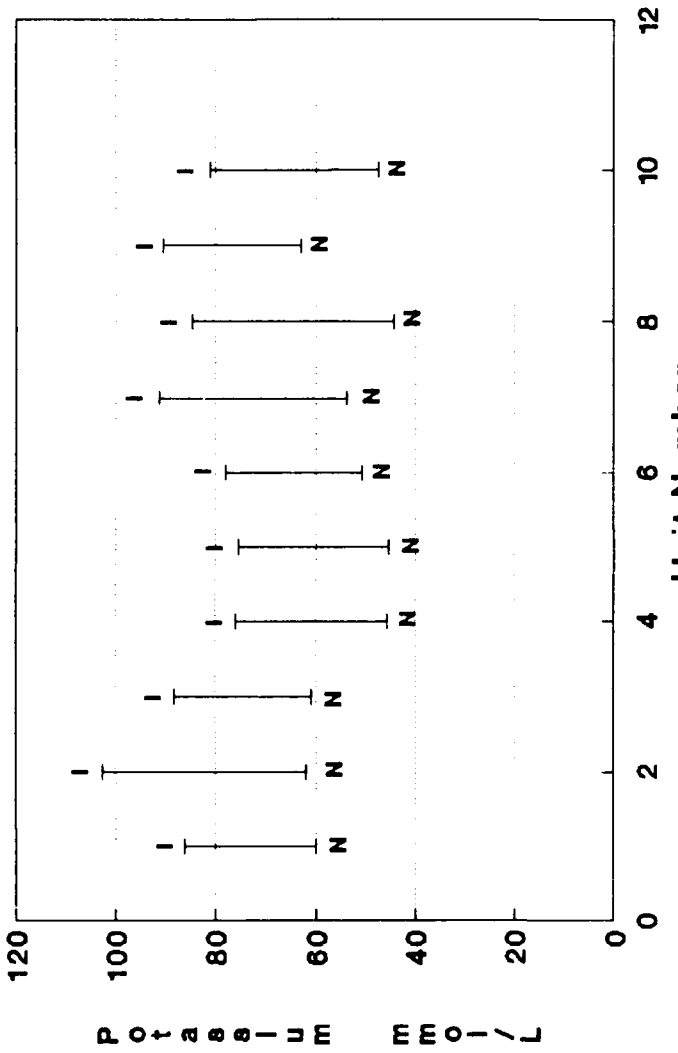
N	Mean	Standard Deviation	Standard Error	t Statistic	P Value
10	36.710	4.518	1.429	25.689	<0.001

FIGURE 6. Comparison of potassium values in mmol/L of individual aliquot pairs of irradiated (I) and non-irradiated (N) CPDA-1 anticoagulated human packed RBCs on day 14 after exposure to 3000 rads of gamma radiation and the statistics of the difference.



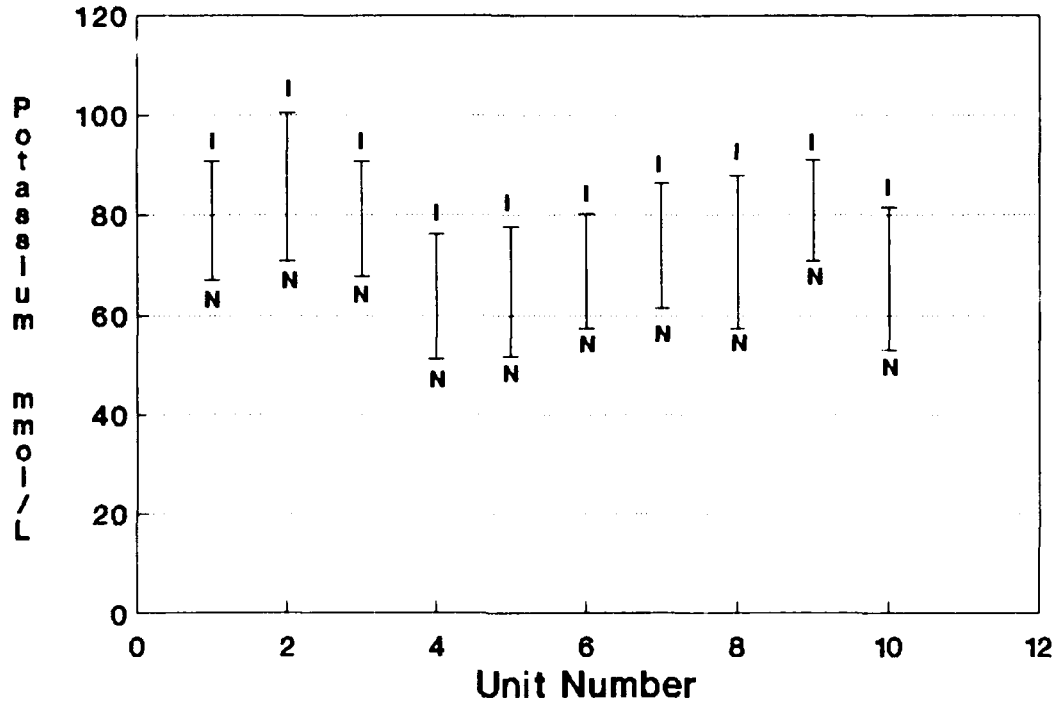
N	Mean	Standard Deviation	Standard Error	t Statistic	P Value
10	36.440	3.653	1.155	31.550	<0.001

FIGURE 7. Comparison of potassium values in mmol/L of individual aliquot pairs of irradiated (I) and non-irradiated (N) CPDA-1 anticoagulated human packed RBCs on day 21 after exposure to 3000 rads of gamma radiation and the statistics of the difference.



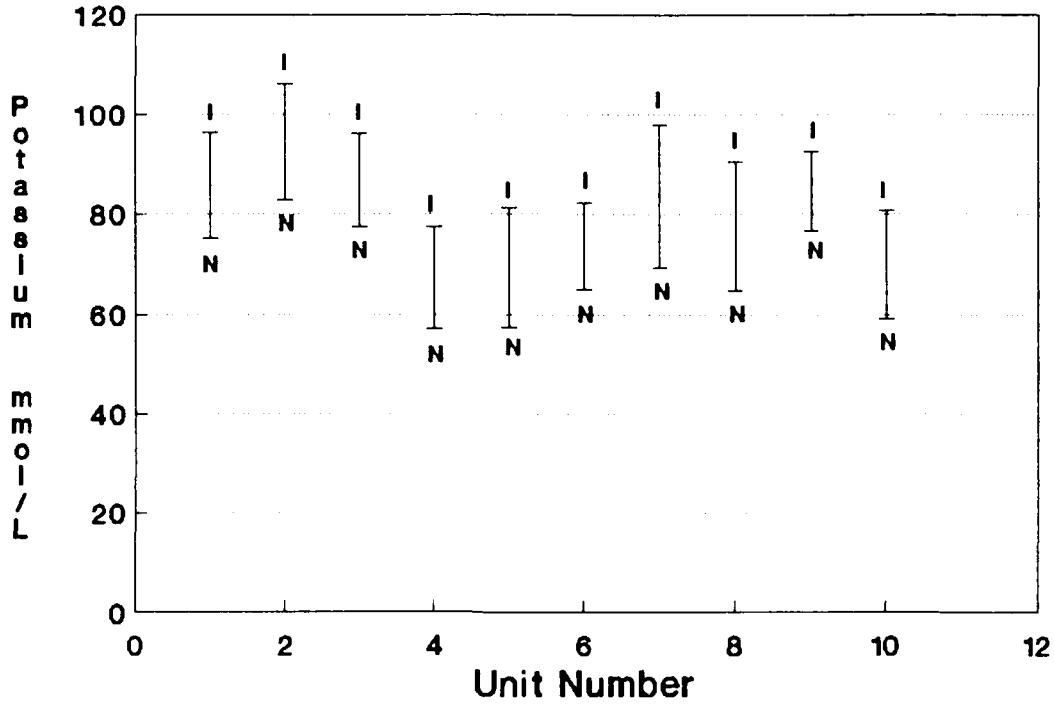
N	Mean	Standard Deviation	Standard Error	t Statistic	P Value
10	32.130	5.611	1.774	18.112	<0.001

FIGURE 8. Comparison of potassium values in mmol/L of individual aliquot pairs of irradiated (I) and non-irradiated (N) CPDA-1 anticoagulated human packed RBCs on day 28 after exposure to 3000 rads of gamma radiation and the statistics of the difference.



N	Mean	Standard Deviation	Standard Error	t Statistic	P Value
10	25.470	3.263	1.032	24.680	<0.001

FIGURE 9. Comparison of potassium values in mmol/L of individual aliquot pairs of irradiated (I) and non-irradiated (N) CPDA-1 anticoagulated human packed RBCs on day 35 after exposure to 3000 rads of gamma radiation and the statistics of the difference.



N	Mean	Standard Deviation	Standard Error	t Statistic	P Value
10	21.670	3.893	1.231	17.604	<0.001

FIGURE 10. Mean differences and the standard errors of potassium values between irradiated and non-irradiated aliquot pairs of CPDA-1 anticoagulated human packed RBCs over 35 days storage. Irradiated aliquots were exposed to 3000 rads gamma radiation prior to storage.

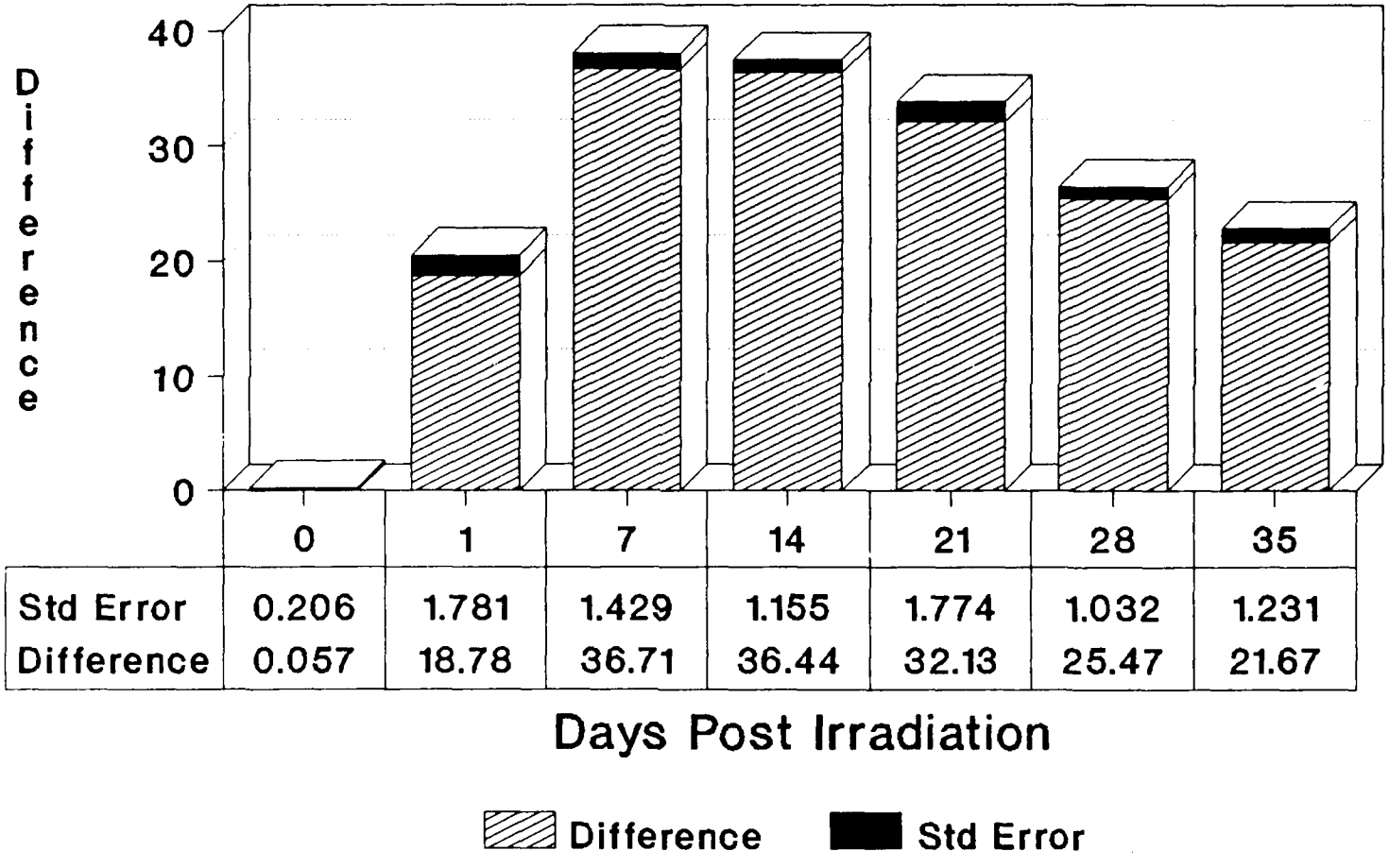

Difference
40
30
20
10
0
0
1
7
14
21
28
35
Std Error 0.206 1.781 1.429 1.155 1.774 1.032 1.231
Difference 0.057 18.78 36.71 36.44 32.13 25.47 21.67
Days Post Irradiation
Difference
Std Error

Table 6

Preinfusion data on the foxhounds. This information was used for 24 hour RBC survival calculations.

Dog No.	Weight	Calculated Blood Volume	Preinfusion Hematocrit	Calculated RBC Mass
1	54.0 lbs	2049.4 ml	34%	627.1 ml
2	56.0 lbs	2128.9 ml	36%	689.8 ml
3	54.0 lbs	2049.4 ml	37%	682.5 ml
4	65.0 lbs	2467.7 ml	29%	644.1 ml
5	63.0 lbs	2392.4 ml	39%	839.7 ml

hematocrit and blood volume data were used to calculate each animal's RBC mass. All phlebotomies and reinfusions were completed without any adverse animal reactions; all animals were returned to Walter Reed Army Institute of Research upon completion of the project.

The volume of labeled autologous blood reinfused ranged from 60 to 82 ml, the hematocrit of the labeled blood ranged from 57% to 77%; and the RBCs labeled ranged from 53.1% to 80.1% (Table 7).

Dog 1 acted as the control for the study; the red cells infused were not irradiated prior to storage. Labeling was performed in the same manner as dogs 2, 3, 4 and 5. Labeled RBCs started at 3.60% at 3 minutes and gradually declined to 2.90% at 1 week (Table 8). The mean channel fluorescence difference between population one (unlabeled RBCs) and population two (labeled RBCs) was greater than 100 channels on all timed specimens.

Dogs 2, 3, 4 and 5 were the test animals, infused with blood irradiated prior to storage. Labeled RBCs for dog 2 peaked at 2.97% at 7.5 minutes and declined to 2.60% at 1 week (Table 9). Peak labeled RBCs for dog 3 was 3.23% at 10 minutes and declined to 2.13% at 1 week (Table 10). Dog 4 labeled RBCs peaked at 5.23% at 3 minutes and declined to 1.93% by 1 week (Table 11). Peak labeled RBCs for dog 5 was 3.07% at 3 minutes and declined to 2.00% by 1 week (Table 12). Mean channel fluorescence difference between populations one and two were greater than 100 channels

Table 7

Preinfusion data of the fluorescent labeled autologous blood transfused to the dogs. These data were used for 24 hour RBC survival calculations.

Dog No.	Blood Treatment	Volume Reinfused	Hematocrit	Percent RBCs Labeled
1	Non-Irradiated	60 ml	77%	65.2%
2	Irradiated	75 ml	68%	75.2%
3	Irradiated	70 ml	77%	53.1%
4	Irradiated	73 ml	69%	71.0%
5	Irradiated	82 ml	57%	80.1%

Table 8

Flow cytometry results of pre- and post-infusion specimens for dog 1, the control. Dog 1 received labeled blood that had not been exposed to gamma radiation prior to 14 days storage. Population 1 is the unlabeled RBCs and population 2 is the labeled RBCs.

	Percentage of Labeled RBCs	Mean Channel Fluorescence	
		Population 1	Population 2
Prelabeled Unit	0.00	12.02	------
Labeled Unit	65.17	16.33	141.33
Preinfusion Dog	0.03	11.64	57.75
3 minutes	3.60	11.61	132.78
5 minutes	3.20	11.72	133.19
7.5 minutes	3.43	11.53	130.43
10 minutes	3.37	11.53	134.98
12.5 minutes	3.17	11.91	135.38
15 minutes	3.23	12.01	130.86
30 minutes	3.00	11.98	133.86
60 minutes	3.20	12.08	134.42
24 hours	3.20	11.91	134.31
1 week	2.90	14.12	139.10

Table 9

Flow cytometry results of pre- and post-infusion specimens for dog 2. Dog 2 received labeled red blood cells that had been exposed to 3000 rads of gamma radiation prior to 14 days storage. Population 1 is the unlabeled RBCs and population 2 is the labeled RBCs.

	Percentage of Labeled RBCs	Mean Channel Fluorescence	
		Population 1	Population 2
Prelabeled Unit	0.03	10.41	48.82
Labeled Unit	75.17	20.26	134.26
Preinfusion Dog	0.1	9.61	53.61
5 minutes	2.83	9.57	127.48
7.5 minutes	2.97	9.60	127.28
10 minutes	2.83	9.69	125.49
12.5 minutes	2.77	9.53	126.73
15 minutes	2.60	9.68	126.35
30 minutes	2.60	9.88	127.45
60 minutes	2.67	9.74	126.74
24 hours	2.80	9.57	122.20
1 week	2.60	9.44	115.02

Table 10

Flow cytometry results of pre- and post-infusion specimens for dog 3. Dog 3 received labeled red blood cells that had been exposed to 3000 rads of gamma radiation prior to 14 days storage. Population 1 is the unlabeled RBCs and population 2 is the labeled RBCs.

	Percentage of Labeled RBCs	Mean Channel Fluorescence	
		Population 1	Population 2
Prelabeled Unit	0.00	10.37	60.00
Labeled Unit	53.10	18.72	133.80
Preinfusion Dog	0.7	12.83	65.64
3 minutes	3.03	12.82	125.31
5 minutes	3.00	12.94	126.77
7.5 minutes	3.07	13.01	131.06
10 minutes	3.23	12.97	124.43
12.5 minutes	3.10	12.97	128.17
15 minutes	2.90	12.95	125.50
30 minutes	2.93	13.09	126.15
60 minutes	2.87	13.02	119.21
24 hours	2.73	13.14	123.41
1 week	2.13	9.55	126.83

Table 11

Flow cytometry results of pre- and post-infusion specimens for dog 4. Dog 4 received labeled red blood cells that had been exposed to 3000 rads of gamma radiation prior to 14 days storage. Population 1 is the unlabeled RBCs and population 2 is the labeled RBCs.

	Percentage of Labeled RBCs	Mean Channel Fluorescence	
		Population 1	Population 2
Prelabeled Unit	0.00	14.78	57.75
Labeled Unit	71.00	24.59	134.94
Preinfusion Dog	0.83	12.19	61.94
3 minutes	5.23	12.05	129.89
5 minutes	4.97	12.14	130.86
7.5 minutes	3.80	12.14	131.39
10 minutes	3.47	12.17	129.32
12.5 minutes	3.40	12.03	129.46
15 minutes	3.00	11.93	127.41
30 minutes	2.17	11.98	125.67
60 minutes	2.30	12.12	119.41
24 hours	2.70	11.99	114.04
1 week	1.93	11.35	138.10

Table 12

Flow cytometry results of pre- and post-infusion specimens for dog 5. Dog 5 received labeled red blood cells that had been exposed to 3000 rads of gamma radiation prior to 14 days storage. Population 1 is the unlabeled RBCs and population 2 is the labeled RBCs.

	Percentage of Labeled RBCs	Mean Channel Fluorescence	
		Population 1	Population 2
Prelabeled Unit	0.00	10.84	49.84
Labeled Unit	80.10	19.70	133.72
Preinfusion Dog	0.03	31.55	47.35
3 minutes	3.07	10.58	131.51
5 minutes	2.97	10.38	132.12
7.5 minutes	2.90	10.42	133.09
10 minutes	2.57	10.49	132.37
12.5 minutes	2.50	10.63	129.78
15 minutes	2.33	18.86	130.46
30 minutes	2.20	10.88	129.84
60 minutes	2.10	10.94	130.55
24 hours	2.37	10.31	123.69
1 week	2.00	10.78	130.83

on all of the timed specimens for each dog.

The percentage of labeled RBCs at time zero must be estimated from blood volume calculations or back extrapolated from early time measurements. Data from three methods of estimating the percentage of labeled RBCs at time zero were evaluated (Table 13). Values calculated from estimates of the animal's RBC mass ranged from 4.2% to 5.6%. Values were lower for the linear regression determinations except in animal 4; values ranged from 3.1% to 6.12%. Logarithmic regression values were very similar to those of the linear regression; values ranged from 3.34% to 6.47%. Peak observed values were reviewed with the other points to evaluate the validity of the calculated points.

The percentage of autologous labeled RBCs surviving 24 hours after reinfusion for each of the three methods used to calculate zero time values was examined (Fig. 11). Dog 1 was the control animal with RBC survivals that ranged from 60% to 87.4%. Red cell survivals for dog 2 had a small range 65% to 70%. Survival of RBCs for dog 4 was much lower by all three methods, ranging from 44.1% to 48.2%. Red cell survivals for dog 5 ranged from 52.6% to 71.6%. The relationship between the different calculation methods was similar in all animals except dog 4; the survival calculation from the red cell mass estimates was higher than those from the back extrapolations.

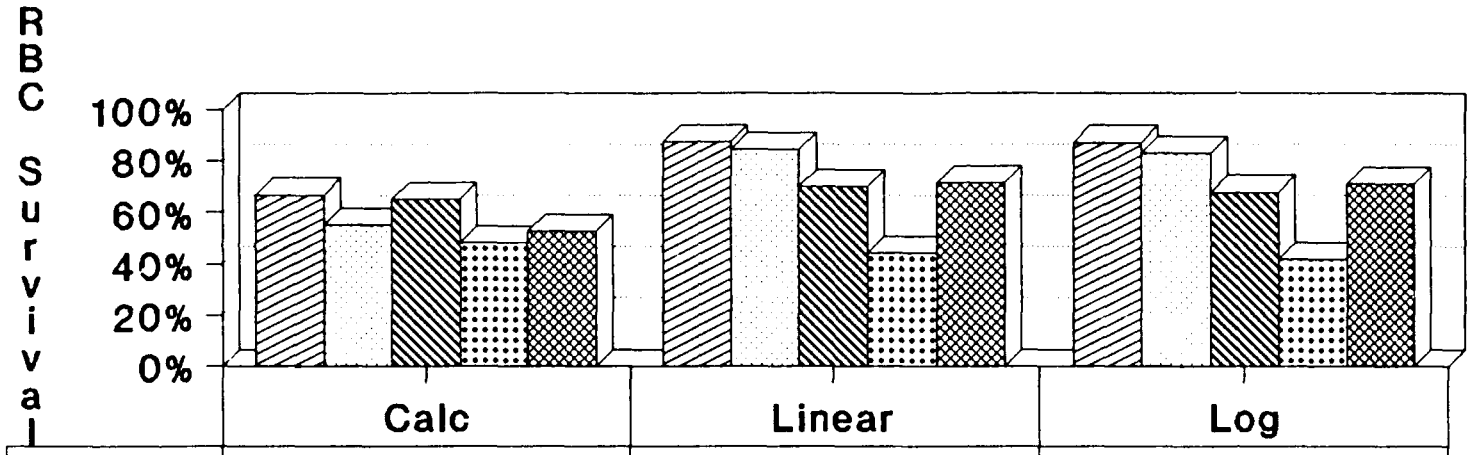
Expected percentage of labeled RBCs surviving 1 week after reinfusion were

Table 13

Percentage of labeled RBCs at time zero. Values were determined using three different methods and listed with the peak observed value.

	Percentage of Labeled RBCs			
Dog No.	Calculated From Weight	Linear Regression	Logrithmic Regression	Peak Value Observed
1	4.8	3.66	3.67	3.60
2	5.6	3.32	3.37	2.97
3	4.2	3.90	4.03	3.23
4	5.6	6.12	6.47	5.23
5	4.5	3.31	3.34	3.07

FIGURE 11. Percentage of autologous labeled RBCs surviving 24 hours after reinfusion in dogs. Values were calculated from blood volume estimates and determined from linear and logarithmic regression analysis. Dog 1 was the control; the red cells were not irradiated prior to 14 days storage. Dogs 2, 3, 4 and 5 received red cells that had been irradiated with 3000 rads of gamma radiation prior to storage for 14 days.



	Calc	Linear	Log
1	66.7%	87.4%	87.2%
2	55%	84.3%	83.1%
3	65%	70%	67.8%
4	48.2%	44.1%	41.7%
5	52.6%	71.6%	71%

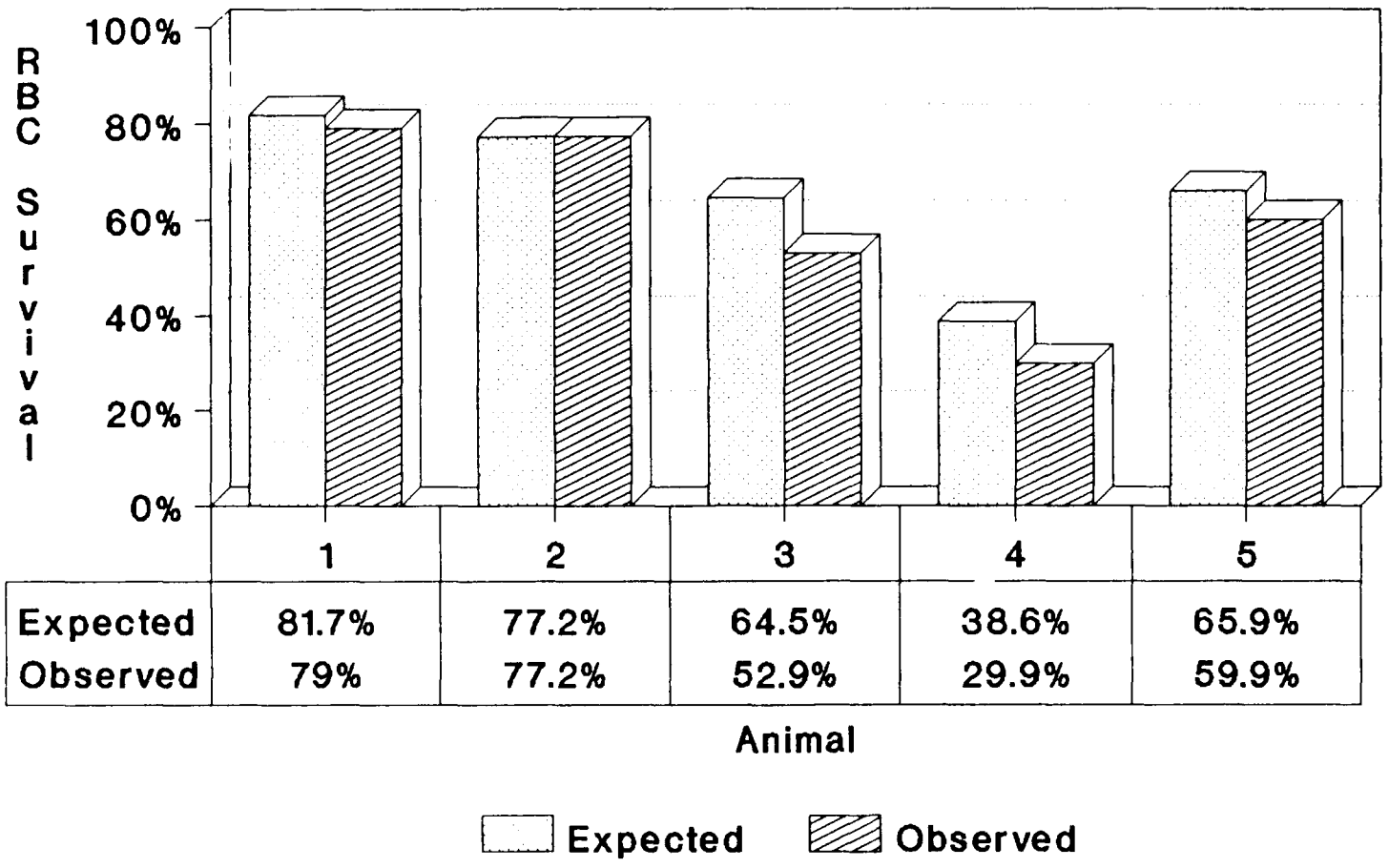
Calculation Method

Animal

1 2 3 4 5

compared to that observed (Fig. 12). The observed RBC survival was equal or smaller than the expected survival in each animal. The expected 1 week red cell survival peaked at 81.7% in animal 1 and ranged down to 38.6% in animal 4. The observed red cell survival peaked at 79.0% in animal 1 and ranged down to 29.9% in animal 4.

FIGURE 12. Expected and observed percentage of autologous labeled RBCs surviving 1 week after reinfusion in dogs. Values were calculated using 24 hour labeled RBC percentage and logarithmic regression analysis zero time red cell values. Dog 1 was the control; the red cells were not irradiated prior to 14 days storage. Dogs 2, 3, 4 and 5 received red cells that had been irradiated with 3000 rads of gamma radiation prior to storage for 14 days.


RBC Survival
100%
80%
60%
40%
20%
0%
1
2
3
4
5
Expected 81.7% 77.2% 64.5% 38.6% 65.9%
Observed 79% 77.2% 52.9% 29.9% 59.9%
Animal
Expected
Observed

DISCUSSION

The results of the *in vitro* study displayed a remarkable contrast in potassium release from irradiated and non-irradiated RBCs. Potassium values of irradiated aliquots were significantly elevated from the first day after irradiation ($p < 0.001$) with a pronounced rise in the potassium levels during the first week. The potassium levels of the irradiated aliquots remained at about twice the level of the non-irradiated aliquots for the first 2 weeks. The leveling of potassium values in irradiated aliquots observed at two weeks was most likely the result of an equilibrium reached between the intracellular and extracellular potassium compartments of the RBCs. The non-irradiated aliquots continued a steady rise of potassium levels because they did not reach the equilibrium point during the 35 day testing period.

The hypothesis that plasma potassium levels of irradiated units of RBCs stored more than 7 days would be elevated was supported, but the size of the rise at 1 day was larger than expected. The rate of increase in potassium levels the first week of storage was larger than expected. Further studies are needed to study the specific membrane changes occurring.

The rapid increase in potassium values of the irradiated RBCs indicate immediate damage of unknown type to the RBC membrane causing increased

potassium leakage. Both the irradiated and non-irradiated aliquots were affected by the expected inhibition of the active transport of potassium back into the red cell. Several researchers have noted RBC membrane changes with various doses of gamma radiation;[70-73] most consistently noted were the formation of hydroperoxides of the membrane lipids[71,72] and disulfide bridging between proteins.[21,71] These studies used very high doses of radiation as compared to those used for prevention of GVHD; it is not known if these changes occur at the lower radiation doses.[70] It has been well accepted that red cell cation transport changes with exposure to gamma radiation, and in all cases there was a decrease in potassium transport[21,70-72,74] resulting in increased extracellular potassium. Some of these changes act like accelerated aging as metabolic depletion results in very similar cation changes.[21]

The active transport inhibition of the normal red cell storage lesion is reversible,[23] but it has not been demonstrated whether the changes that occur as a result of radiation are reversible. Refrigeration may inhibit repair mechanisms in a similar manner as the sodium-potassium pump allowing the effects of irradiation to become more apparent. It is not known if the sodium-potassium pump can overcome the radiation induced membrane damage once transfused.

Removing the plasma of irradiated blood and replacing it with saline prior to transfusion would remove the majority of the extracellular potassium,[59]

but for exchange transfusions there would then be a need for replacing the removed plasma with donor plasma and exposing the neonate to an additional blood donor.

Clinical significance of the increased potassium is difficult to assess. The total potassium content of the volume of blood transfused needs to be evaluated[22,60] as well as the acute impact of the transfused potassium. Ten ml of 7 day old irradiated blood would contain approximately 0.2 mmol of extracellular potassium which would be initially diluted by the entire blood volume and eventually by the body water volume of the recipient.[22] Under normal conditions this amount of potassium would not be a problem unless compounded by other circumstances. Massive or frequent transfusions may not be so easily tolerated, especially by patients with impaired renal function. Neonates would be particularly affected because of their small blood volume and poorer compensation mechanisms; infants receiving blood transfusions are usually sick premature infants that have a poor ability to compensate. To determine clinical significance of potassium elevation in stored irradiated units of blood the physician needs to be informed of the elevated potassium content of the blood products being provided so that all of the factors for each patient can be considered.

Previous studies report red cell survival following irradiation was

normal,[52] but survival was not evaluated after irradiation and storage. In this study *in vivo* 24 hour RBC survival was not clearly affected by irradiation and storage. All irradiated units had lower 24 hour RBC survivals than the control by all three calculation methods. All irradiated unit survivals were near or below 70% at 14 days storage. All observed results from the 1 week RBC survival study were equal to or lower than those expected. The small difference between the observed and expected survival seen in dog 1, the control, could be the result of a loss of red cells slightly damaged during the labeling process or individual variation.

The fluorescent labeling method provided excellent results. Staining quality was demonstrated by the large mean channel fluorescence difference between the labeled and unlabeled cell populations. Cells were clearly labeled or not labeled with no overlap of the two populations. The dye was stable throughout the procedure and samples stored in the dark retained their fluorescence. The 1 week samples continued to display large mean channel differences indicating no loss of the label from the RBCs.

Early sample points of dogs 1, 2, and 3 were not peak values and were very inconsistent. There appeared to be delayed mixing of the labeled blood in the circulation in dogs 1, 2 and 3. Early data points in dogs 4 and 5 were stable, most likely a result of their larger size.

Logarithmic regression analysis is the recommended method to evaluate data points for RBC survival;[63,64,75] however, little difference was observed between it and the linear regression analysis results in the present study. The method with the greatest error is the survival calculations using the RBC mass estimate since the blood volume is based on figures generalized for all dogs.

The low red cell survivals observed in dog 4 are probably the result of individual variation observed in survival study procedures.[64,65] Paired studies using the same research subject for infusion of both irradiated and non-irradiated samples would remove individual variability[65] but requires sufficient time between the two procedures for all labeled cells to be cleared from the subject's circulation.

The labeling process causes some RBC loss as a result of hemolysis and cells coating plasticware, but it was not possible to quantify. The degree of visual hemolysis related more to the degree of labeling than irradiation status. Measurements of blood volume and hematocrit showed no difference in red cell recoveries between irradiated and non-irradiated units at labeling. The labeling process is known to damage the RBC membrane, although insufficiently to cause premature removal from the circulation.[66] The trauma of the labeling in addition to the radiation

membrane damage may have caused some decrease in recovery and survival. It is possible that red cells severely damaged by radiation were lost in the labeling process and never considered in the survival calculations. This would result in falsely elevated RBC survivals.

The hypothesis that irradiation would have no effect on RBC survival was not clearly supported. Results indicate some affect on RBC survival and results warrant additional studies to further investigate longer term survivals and explore the accelerated aging theory. Membrane damage studies using sensitive methods are indicated. Paired chromium 51 studies in humans should be performed to verify the animal results.

Results from both *in vitro* and *in vivo* studies indicate irradiation damages RBCs, affecting them in storage and after transfusion. The effects are not so large as to allow a clear definition of what should be permitted for all patients. Graft versus host disease is a serious risk in certain patients and the high fatality rate warrants irradiated blood for those patients even if it must be stored prior to transfusion. It is not economically feasible for every facility to have an irradiator so it is inevitable that compromises will result. The benefits and risks of blood irradiation need to be evaluated for each patient and some of the decision responsibility given to the patient. When family member blood donors are requested by a patient, the additional risks of GVHD and blood product deterioration caused by irradiation should be thoroughly

discussed. It cannot be determined which is the greater risk, transfusion transmitted disease, GVHD or radiation damaged RBCs. Blood products should not be indiscriminately irradiated; storage after irradiation should be allowed only when there is no other alternative.

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APPENDIX A

In Vitro Labeling Experiments

Dye Concentration Experiment

Methods

The purpose of this experiment was to determine the lowest concentration of dye (PKH26, Zynaxis Cell Science Inc.) providing satifactory labeling. One hundred ul specimens of dog blood were labeled with 5×10^{-6} M, 10×10^{-6} M, 15×10^{-6} M, and 20×10^{-6} M dye concentrations. For ease of preparation, 500 ul of stock 20×10^{-6} M dye was made from the dye concentrate and other dye concentrations were made by dilution using the following formulas.

a. $5 \times 10^{-6}\ M \times 100\ ul = 20 \times 10^{-6}\ M \times X$

$= 25\ ul$

b. $10 \times 10^{-6}\ M \times 100\ ul = 20 \times 10^{-6}\ M \times X$

$= 50\ ul$

c. $15 \times 10^{-6}\ M \times 100\ ul = 20 \times 10^{-6}\ M \times X$

$= 75\ ul$

d. $20 \times 10^{-6}\ M \times 100\ ul = 20 \times 10^{-6}\ M \times X$

$= 100\ ul$

The following formula was used to calculate volume of dye required:

$20 \times 10^{-6}\ M \times 500\ ul = 1 \times 10^{-3}\ M \times X$

$= 10\ ul$

All labeling was performed following the same procedure. Seventy-one ul of packed red cells from a non-irradiated dog specimen, 7 days old were

used. Normal dog hematocrit was estimated to be 50% and the blood specimen hematocrit was 71%. Blood was washed twice with sodium chloride injection, 0.9%, USP (Kendall McGaw Laboratories, Inc.), to remove plasma proteins. Diluted dye was added and incubated for 4 min. This labeling time was based on previous work. Cells were washed three times with 1%, vol/vol, solution of fetal bovine serum (Gibco Laboratories, Life Technologies, Inc.) to stop the staining action. The fetal bovine serum was heat inactivated for 30 min at 56° C and diluted in sodium chloride irrigation, 0.9%, USP, (Kendall McGaw Laboratories, Inc.). Cells were then washed twice with sodium chloride injection, 0.9%, USP (Kendall McGaw Laboratories, Inc.) to remove the fetal bovine serum.

Five ul of the labeled and unlabeled blood were diluted in 1 ml phosphate buffered saline (American Scientific Products) and analyzed on the Becton Dickinson FACScan flow cytometer (Becton Dickinson Immunocytochemistry Systems). FSC (forward light scatter detector) and SSC (side light scatter detector) settings were changed to log and the flow rate changed to low. Ten thousand cells were analyzed for each sample and each specimen was analyzed in triplicate. Using Consort 30 data analysis software (Becton Dickinson Immunocytochemistry Systems, Mountain View, CA) graphs were generated for each sample demonstrating forward light scatter versus side light scatter and fluorescence emission on the FL2 detector. Labeled and unlabeled red blood cell (RBC) populations were graphically separated and the number of labeled cells and intensity of the

fluorescence quantified on the FL2 channel. Percentage of RBCs labeled and the mean channel of fluorescence were compared for the different concentrations.

Results

There was a pronounced increase in the mean channel fluorescence to 120 at dye concentration of 5×10^{-6} M with only a small increase at higher dye concentrations (Fig.13A). There is an increase in the RBCs labeled to 60% at a dye concentration of 5×10^{-6} M and an additional increase to over 80% at a dye concentration of 10×10^{-6} M with essentially no change with higher dye concentrations (Fig. 13B).

Discussion

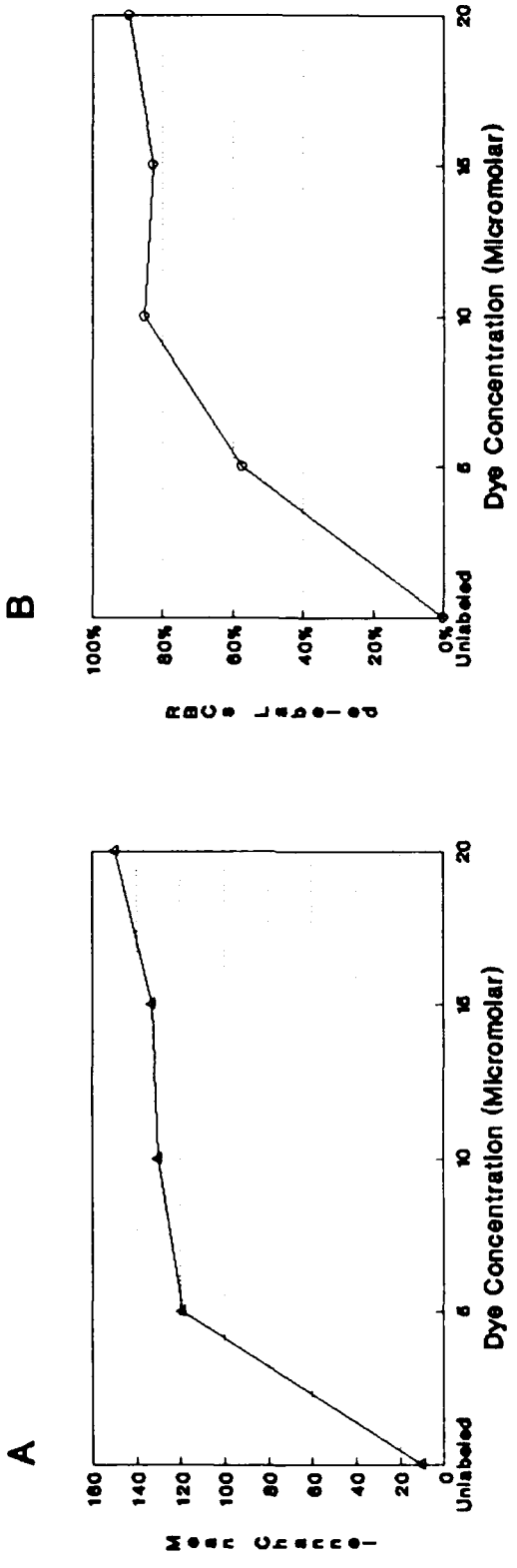
Dye concentration of 10×10^{-6} M was selected for use in the *in vivo* portion of the study based on the fluorescence intensity as indicated by the mean channel values and the peak in the percentage of red cells labeled. The small improvements observed for the higher dye concentrations do not justify the cost of the additional dye required.

Dye Incubation Time Experiments

Methods

The purpose of this experiment was to determine the labeling time with the highest percentage of adequately labeled cells with minimal hemolysis. The blood used was from a specimen of non-irradiated dog blood, stored for

FIGURE 13. Fluorescence intensity and percentage of labeled RBCs for different dye concentrations using packed RBCs from a non-irradiated dog specimen, 7 days old. All samples were incubated for 4 min at room temperature. A. Mean channel of fluorescence for the different dye concentrations. B. Percentage of RBCs labeled for the different dye concentrations.

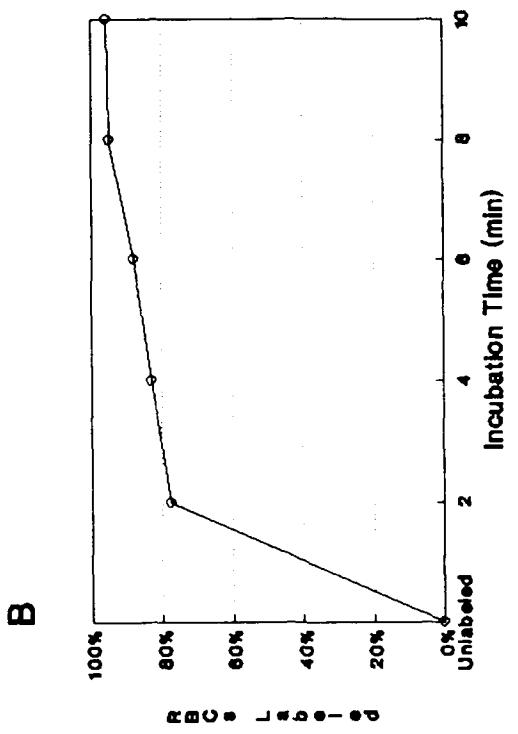

A
Mean Channel
180
140
120
100
80
60
40
20
0
Unlabeled
5
10
15
20
Dye Concentration (Micromolar)
B
RBCs Labeled
100%
80%
60%
40%
20%
0%
Unlabeled
5
10
15
20
Dye Concentration (Micromolar)

7 days; the experiment was later repeated using irradiated dog blood 10 days old. Labeling times studied were 2, 4, 6, 8 and 10 minutes. All labeling was performed at a 10 x 10^{-6} M dye concentration. All washing steps were performed as previously described in the dye concentration experiment. All supernates from the first and second washes were collected and examined spectrophotometrically at 410 nm to measure hemoglobin release from damaged RBCs. Labeled cells were tested on the flow cytometer as previously described to examine labeling efficiency as percentage of cells labeled and mean channel of fluorescence.

Results

The mean channel fluorescence of the non-irradiated blood rose to 90 and did not increase with the longer incubations (Fig. 14A). The percentage of non-irradiated red cells labeled rose to 80% at the 2 minute incubation with a steady increase to 90% by the 8 minute incubation (Fig. 14B). The mean channel fluorescence of the irradiated blood rose to 120 and did not increase with the longer incubations (Fig. 15A). The percentage of irradiated RBCs labeled was 90% at 2 minutes and did not change with longer incubations (Fig. 15B). There was not a large difference in absorbances of the washes at the various incubation times with the non-irradiated blood (Fig. 16A). There absorbance of the wash with the irradiated blood peaked at the 8 minute incubation and dropped at 10 minute incubation (Fig 16B). Absorbances from both 2 minute washes and the 4 minute first wash were not available because of mechanical problems.

FIGURE 14. Fluorescence intensity and percentage of labeled RBCs for different incubation times using packed RBCs from a non-irradiated dog specimen, 7 days old. Dye concentration of 10 x 10^{-6} M was used for all tests. A. Mean channel of fluorescence for the different incubation times. B. Percentage of RBCs labeled for the different incubation times.


B
100%
80%
60%
40%
20%
0%
Unlabeled
2
4
6
8
10
Incubation Time (min)

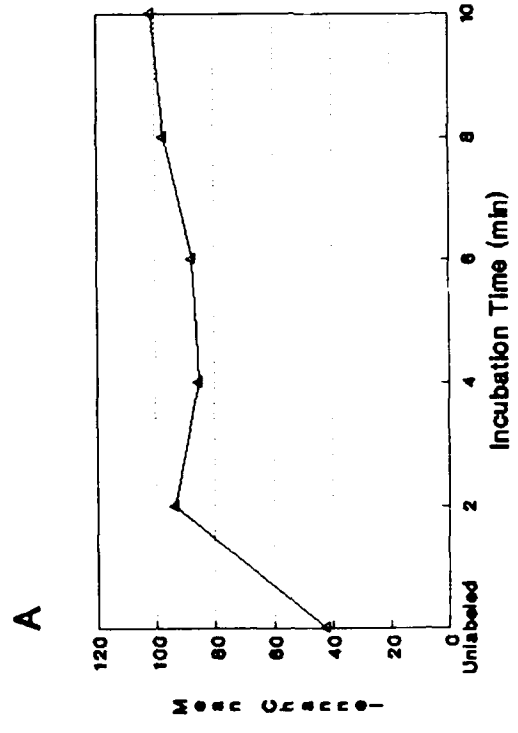
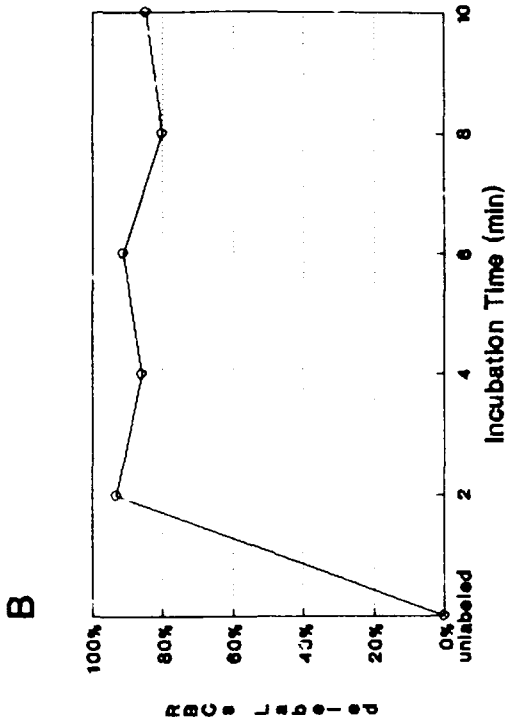

A
120
100
80
60
40
20
0
Unlabeled
2
4
6
8
10
Incubation Time (min)

FIGURE 15. Fluorescence intensity and percentage of labeled RBCs for different incubation times using packed RBCs from a irradiated dog specimen, 10 days old. Dye concentration of 10 x 10^{-6} M was used for all tests. A. Mean channel of fluorescence for the different incubation times. B. Percentage of RBCs labeled for the different incubation times.


B
100%
80%
60%
40%
20%
0%
unlabeled
2
4
6
8
10
Incubation Time (min)

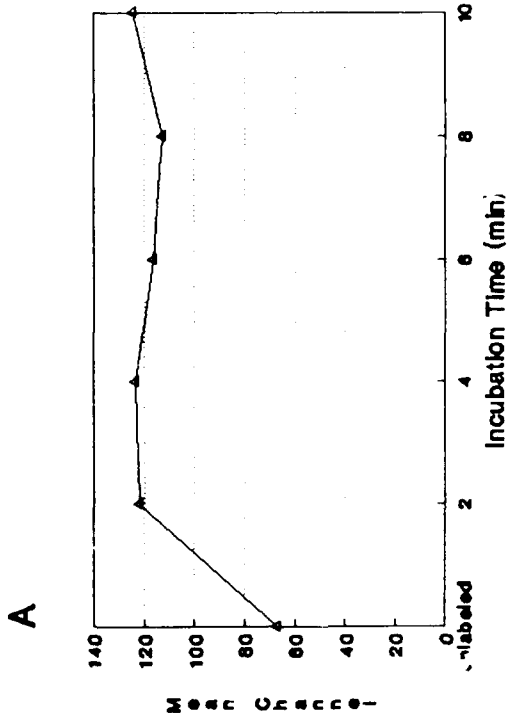
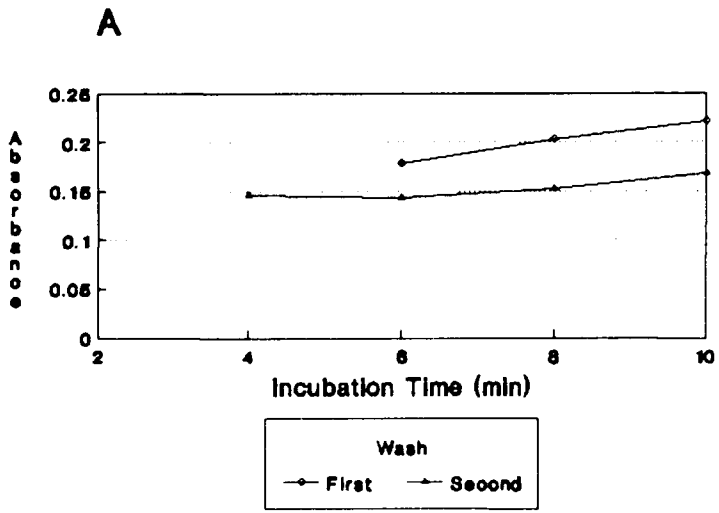
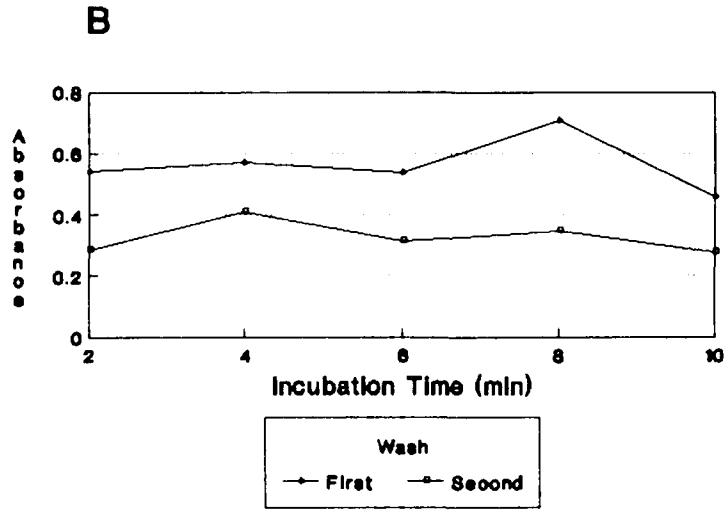

A
140
120
100
80
60
40
20
0
2
4
6
8
10
Incubation Time (min)

FIGURE 16. Absorbance readings at 410 nm of the first and second washes from the incubation time experiment. The dye concentration used was 10×10^{-6} M. A. Absorbance readings from non-irradiated 7 day old dog blood specimen. B. Absorbance readings from irradiated 10 day old dog blood specimen.


A
Absorbance
0.25
0.2
0.15
0.1
0.05
0
2
4
6
8
10
Incubation Time (min)
Wash
First
Second


B
Absorbance
0.8
0.6
0.4
0.2
0
2
4
6
8
10
Incubation Time (min)
Wash
First
Second

Discussion

Longer incubation times clearly did not result in better labeling. Four minutes was chosen as a time in which satisfactory labeling occurred without an increase in hemolysis. It was felt that a two minute timing was too difficult to control accurately. The drop in absorbance at ten minutes observed in the hemolysis data with the irradiated cells could not be explained; it was expected to be as high or higher than the shorter incubation times. The dye manufacturer recommends the shortest incubation time providing adequate labeling intensity as membrane damage can occur with extended incubations or high dye concentrations.